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14. ABSTRACT

Paget's disease (PD) of bone occurs in 3-4% of population over the age of 50. We have identified expression of measles virus nucleocapsid transcripts in osteoclast (OCL) precursors and that MVNP expression induces pagetic phenotype in osteoclasts with increased bone resorption activity as seen in patients with Paget's disease. We previously cloned and identified osteoclast inhibitory peptide-1 (OIP-1/hSca) which inhibits osteoclast formation and bone resorption. We hypothesize that MVNP expression in osteoclast precursors modulates RANK receptor signaling leading to Pagetic OCL development. OIP-1 blocks these signaling events and inhibits MVNP induced osteoclastogenesis and elevated bone resorption activity. We demonstrated that MVNP increases TNF-alpha induced OCL differentiation and activation by increasing NF-kB signaling through increased expression of p62, and IKK-gama and increased MAPK signaling. Our results also suggest that MVNP's effects on TNF-alpha signaling contribute to the increased OCL formation in PD. Furthermore, expression of MVNP gene in OCL in vivo induces a pagetic-like phenotype. RANKL stimulation of OIP-1 mice derived bone marrow cells resulted in significantly decreased osteoclast formation. Furthermore, OIP-1 transgenic mouse bones demonstrated an osteopetrotic phenotype. These data suggest that OIP-1 is an important physiologic regulator of osteoclast development and bone resorption in vivo and may have therapeutic utility to control excess bone turnover in patients with Paget's disease.

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INTRODUCTION:

Paget's disease affects approximately 2-3 million people in the United States and is the second most common bone disease after osteoporosis. We shown that bone marrow cells from patients with Paget's disease express measles virus nucleocapsid protein (MVNP) transcripts and further demonstrated that expression of the Edmonston MVNP gene in normal osteoclast (OCL) precursors results in formation of OCL that share many of the characteristics of OCL from Paget's patients. The MVNP gene contained several sense mutations, which constituted 1% of the nucleotide sequence. The pathologic significance of MVNP and associated mutations to induce abnormal OCL formation and activity in Paget's disease, is unknown (1). RANKL is a member of Tumor necrosis factor (TNF) family member that is expressed on stromal/osteoblast cells and RANK receptor is expressed on committed osteoclast precursor cells. RANKL/RANK signaling is critical for osteoclast differentiation and bone resorption activity in vitro and in vivo (2,3). We have recently cloned and identified the Ly-6 family member, osteoclast inhibitory peptide-1 (OIP-1/hSca) which inhibits osteoclast formation and bone resorption activity. We have further demonstrated that OIP-1 significantly inhibits TNF receptor associated factor-2 (TRAF-2) and c-Jun kinase activity in osteoclast precursor cells (4). Our hypothesis is that MVNP expression in osteoclast precursors modulates the status of RANK receptor signaling molecules leading to Pagetic OCL development in Paget's disease. OIP-1 blocks RANK receptor signaling events and inhibits MVNP induced osteoclastogenesis and elevated bone resorption activity in Paget's patients.

BODY:

The progress on Task-2 (24-36 months) in the statement of work is as follow:

Task 1. Determine the sensitivity of MVNP transduced osteoclast precursors to RANK Ligand (RANKL) and TNF-alpha stimulation to form pagetic osteoclasts (Months 1-24):

Completed

Task 2. Determine the RANK receptor signaling in MVNP transduced osteoclast precursors (Months 24-36).

(a) Examine the status of various RANK adaptor molecules such as TRAFs, p38 MAP kinase activation and JNK activity in MVNP transduced osteoclast precursors in response to RANKL and TNF-alpha stimulation (Months 17-22).

We have examined the effect of measles virus nucleocapsid protein (MVNP) expression on RANK receptor signaling molecules during osteoclast differentiation. As shown in Fig.1, MVNP transduction into human bone marrow derived osteoclast precursors significantly increased the levels of c-Fos and NFATc1. In contrast, there was no significant change in the levels of TRAF2 adaptor protein expression levels in RANKL or TNF-alpha stimulated osteoclast precursor cells compared to empty vector transduced cells.

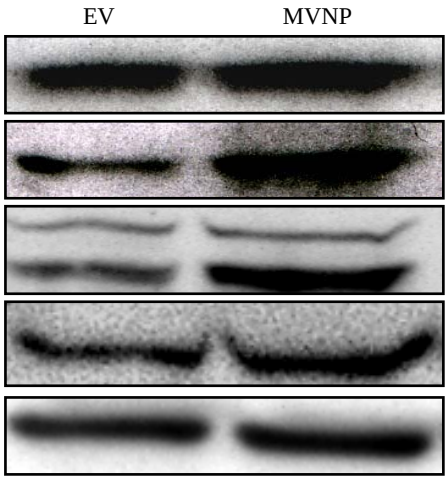


Fig.1 Western blot analysis of RANK signaling molecules in MVNP stimulated osteoclast precursor cells.
Human bone marrow derived non-adherent cells were transdued with or without MVNP gene and treated with hM-CSF (10 ng/ml) and hRANKL (100 ng/ml) for 48 hours. The expression of TRAF2, cFos, NFATc1 and RANK was analyzed. The expression of b-actin in all the samples was analyzed to normalize the protein loading onto the gels.

(b) Determine the NF-κB and AP-1 activation in during osteoclast differentiation of MVNP transduced precursor cells (Months 22-29).

AP-1 complex is formed by c-Jun and c-Fos transcription factors. Therefore we further examined the levels of c-Jun phosphorylation in MVNP transduced osteoclast precursor cells by Western blot analysis. As shown in Fig.2, human bone marrow derived non-adherent mononuclear cells transduced MVNP identified significant increase (2.5 fold) in the levels phospho-c-jun levels in response to RANKL stimulation compared to empty vector transduced cells.

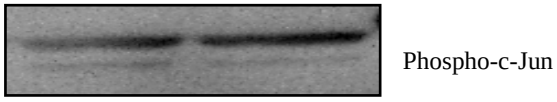


Fig.2. c-Jun phosphorylation analysis of AP-1 complex.
Human bone marrow derived non-adherent cells were transdued with or without MVNP gene and treated with hM-CSF (10 ng/ml) for 48 hours. The preosteoclast cells were treated with hRANKL (100 ng/ml) for 30 mins. The total cell lysate prepared for p-c-Jun kinase activity.

In collaborative studies, we have further examined osteoclast differentiationin TRAP-MVNP transgenic mouse bone marrow cells. As shown in Fig.3, TRAP-MVNP transgenic mouse bone marrow stimulated with RANKL resulted in significantly increased in number and size of osteoclasts compared to wild type mice. Furthermore, MVNP derived from paget's patient also demonstrated similar results. These data suggest that MVNP induce pagetic phenotype in osteoclasts (Kurihara et al JBMR 2006 article copy appended). In related studies, we have also identified high levels expression of kininogen (KNG) in serum samples from patients with paget's disease. KNG had no significant effect on osteoclast stimulation and did not enhance RANKL expression in marrow stromal/preosteoblast cells. However, it does enhance survival of these cells (Eichi et al 2006 article copy appended).

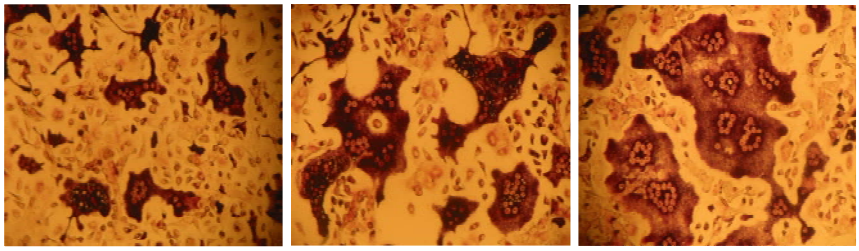
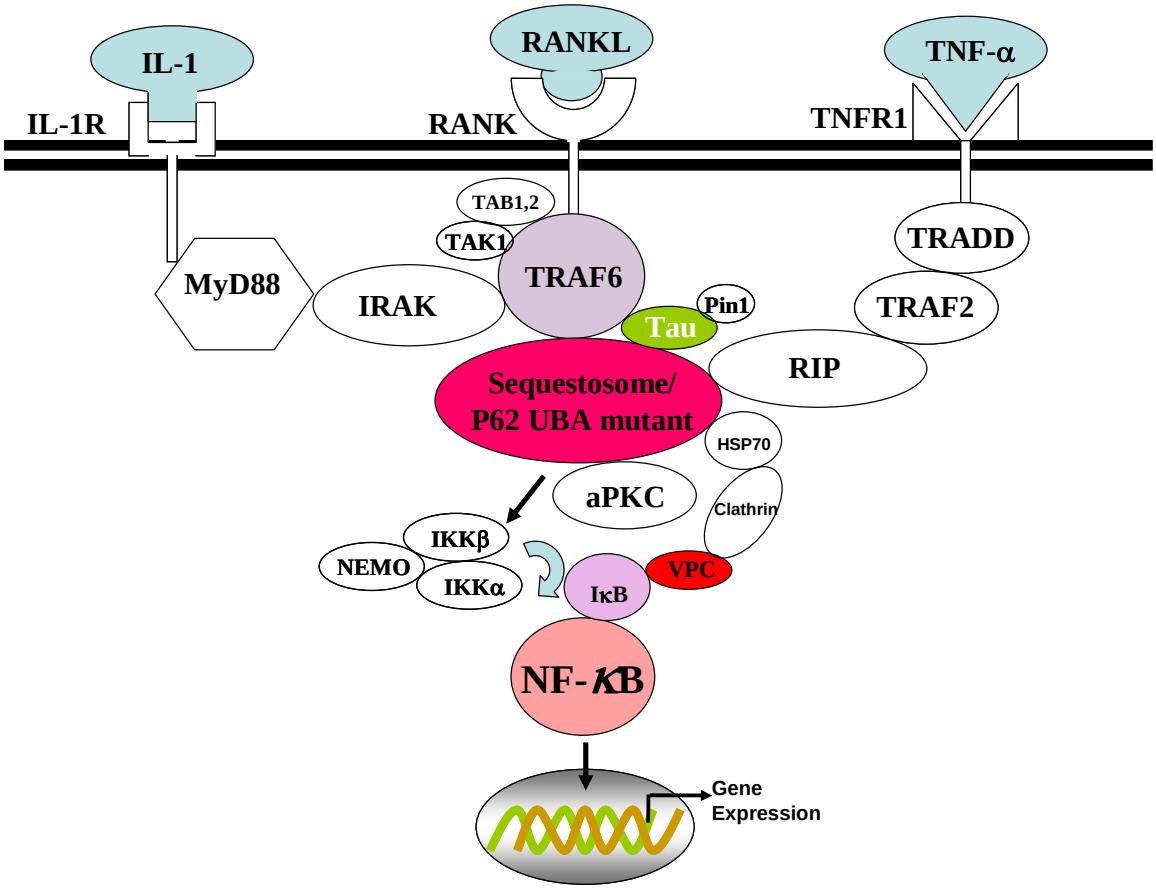


Fig.3. Osteoclast differentiation in MVNP transduced mouse bone marrow cells.

(c) Characterize the role of p62 in RANKL/TNF-alpha stimulated MVNP transduced osteoclast precursor cells (Months 29-36).

We have recently developed a schematic model implicating the role of P62 in RANKL signaling molecules during osteoclastogenesis (Reddy 2006; article copy appended). P62 is mutated in majority of patients with paget's disease. Therefore, we examined the role of MVNP transduction into TRAP-P62 wild type and mutant P62 transgenic mouse bone marrow derived cells in collaborative studies.



As shown in Fig.4, transfection of CFU-GM from wild type (WT) and TRAP-*p62*^{P392L} mice with the MVNP gene further increased levels of NF-κB in TRAP-*p62*^{P392L} osteoclast precursors compared to WT precursors. Expression of MVNP in osteoclast precursors from WT or TRAP-*p62*^{P392L} mice did not increase expression of c-fos. These data suggested that p62 contributes to significantly increased NF-kB activation during osteoclast differentiation in patients with paget's disease.

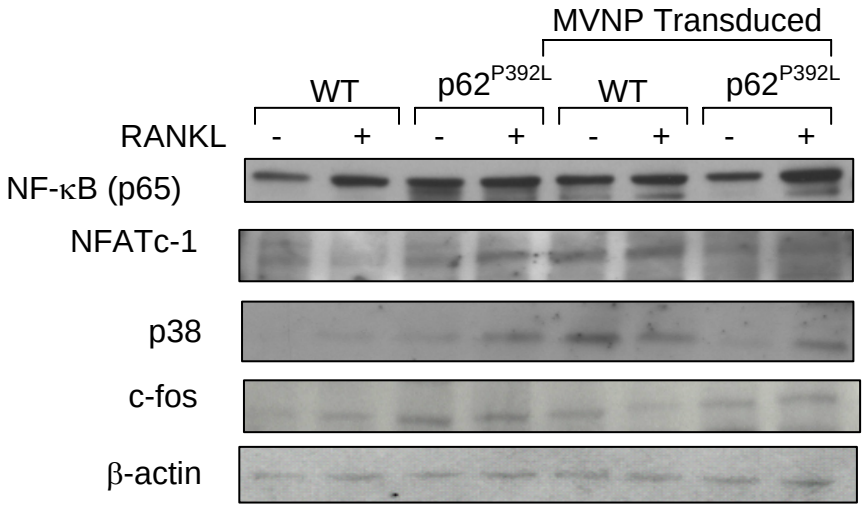


Fig.4 Expression of signaling molecules by osteoclast precursors from MVNP or EV transduced TRAP-p62^{P392L} and WT mice**.** Cell lysates were obtained from day 4 CFU-GM-derived cells and were immunoblotted using anti- NF-κB (p65), NFATc-1, p38MAPK or c-fos rabbit polyclonal antibody.

Task 3. Determine the effects of OIP-1 on MVNP altered RANK receptor signaling in osteoclast precursor cells (Months 29-48).

(a) Determine the effect of OIP-1 on osteoclast differentiation of MVNP transduced osteoclast precursors (Months 29-35).

We have previously cloned and characterized the osteoclast inhibitory peptide-1 (OIP-1) (Ref.4). We now tested the capacity of OIP-1 to inhibit MVNP stimulated osteoclast differentiation and bone resorption activity in human bone marrow cultures. As shown in Fig.5, OIP-1 inhibited MVNP stimulated osteoclast differentiation in human bone marrow cultures in a dose dependent manner. Similarly, OIP-1 significantly bone resorption capacity of MVNP stimulated osteoclasts cultured on hydroxy-appetite (bone substitute) coated plates (Fig.6).

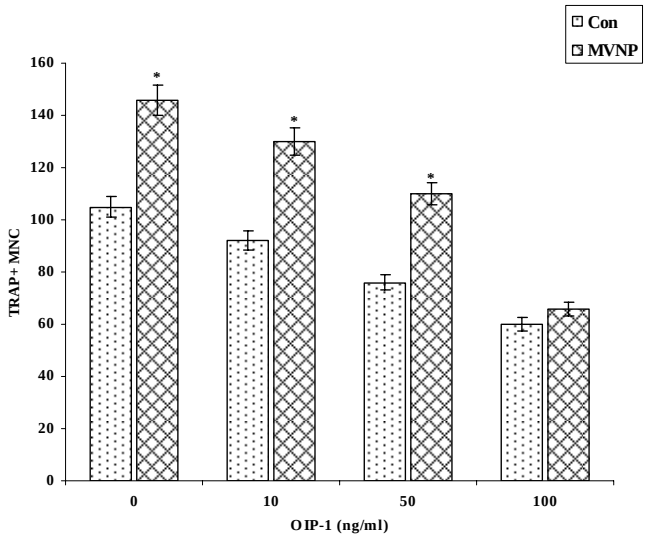


Fig.5. Effect of osteoclast inhibitory peptide-1 (OIP-1) on MVNP stimulated osteoclast differentiation in human bone marrow cultures.

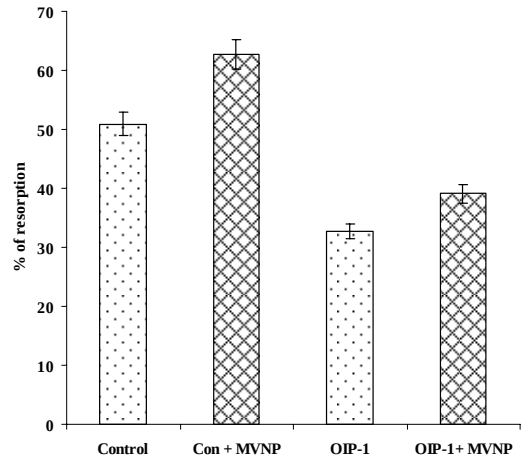


Fig.6. Effect of OIP-1 (100ng/ml) on bone resorption activity in MVNP stimulated osteoclasts.

These results suggest that OIP-1 may have therapeutic utility against MVNP stimulated osteoclast differentiation and bone resorption activity in patients with Paget's disease.

(b) Assess the status of RANK receptor signaling molecules in MVNP stimulated osteoclasts (35-41). -- **Not yet initiated**

(c) Determine the potential of OIP-1 to block MVNP stimulated osteoclast formation and bone resorption in vivo (41-48). -- **Not yet initiated**

KEY RESEARCH ACCOMPLISHMENTS:

- We have identified MVNP transduction will enhance RANK signaling molecules such as c-Fos and NFATc1 during osteoclast differentiation.
- We have shown that P62 enhance NF-kB activation during osteoclast differentiation.
- P62 contributed to enhanced NF-kB activation in pagetic osteoclasts.
- Our data suggest that OIP-1 may have therapeutic utility against excess bone resorption activity in Paget's disease.

REPORTABLE OUTCOMES:

Published articles relevant to the proposal:

1. **Reddy SV**. Etiologic factors in Paget's disease of bone. Cellular Molec. Life Sci. 63:391-398, 2006.
2. Tsuruga E, Rao DS, Baatz JE and **Reddy SV**. Elevated serum Kininogen in patients with Paget's Disease of Bone: A role in marrow stromal/preosteoblast cell proliferation. J Cell. Biochem. 98:1681-88, 2006.
3. Kurihara N, Zhou H, **Reddy SV**, Garcia Palacios V, Subler MA, Dempster DW, Windle JJ, Roodman GD. Expression of measles virus nucleocapsid protein in osteoclasts induces Paget's disease-like bone lesions in mice. J Bone Miner. Res. 21:446-55, 2006.

Abstracts:

1. Tsuruga E, Rao S, **Reddy SV**. Elevated serum high molecular weight kininogen in patients with Paget's disease of bone. 27th ASBMR meeting, 2005, Nashville, TN.

2. Srinivasan S, Tsuruga E, Ries WL, Key Jr LL, Yang S, **Reddy SV**. Interferon-γ regulation of Osteoclast Inhibitory Peptide-1 (OIP-1/hSca) gene promoter activity**.** 27th ASBMR meeting 2005, Nashville, TN.

3. S. Shanmugarajan, K. Irie, W.L. Ries and **S.V. Reddy** Targeting Osteoclast Inhibitory Peptide-1 (OIP-1) Expression to the Osteoclast Lineage in Transgenic Mice Inhibits Osteoclast Formation/Activity. ASBMR, 2006, Philadelphia.

4. Shanmugarajan S, Rao DS and **Reddy SV**. Osteoclast Inhibitory Peptide-1 (OIP-1) Inhibits Measles Virus Nucleocapsid Protein Stimulated Osteoclast Formation/Activity. ASBMR, 2006, Philadelphia.

CONCLUSIONS:

In conclusion, our results demonstrate that MVNP significantly enhanced RANK receptor signaling molecules such as c-Fos and NFATc1. Also, OIP-1 may have therapeutic utility against MVNP stimulated osteoclast activity in patients with Paget's disease.

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1. S.L. Teitelbaum, F.P. Ross, *Nat. Rev. Genet.* **4,** 638 (2003)

2. G. D. Roodman, Windle JJ, *J Clin Invest.* **115,** 200 (2005)

3. J.L. Roccisana *et al., J. Biol. Chem.* **279,** 10500 (2004).

4. M. Koide, H. Maeda *et al., J Bone Miner Res.* **18,** 458 (2003).

APPENDICES:

Attached are reprints for three relevant articles as noted under outcomes.

Cell. Mol. Life Sci. 63 (2006) 391–398
1420-682X/06/040391-8
DOI 10.1007/s00018-005-5473-9

Cellular and Molecular Life Sciences

Visions & Reflections

Etiologic factors in Paget's disease of bone

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Abstract. Paget's disease of bone is a chronic focal skeletal disorder characterized by increased bone resorption by the osteoclasts. Paramyxoviral gene products have been detected in pagetic osteoclasts. Paget's disease is an autosomal dominant trait with genetic heterogeneity. Several mutations in the ubiquitin-associated (UBA) domain of sequestosome 1 (SQSTM1/p62) have been identified in patients with Paget's disease. Similarly, mutations in the valosin-containing protein (VCP) gene have been shown to cause inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia. In addition, gene polymorphisms and enhanced levels of cytokine/growth factors associated with Paget's disease have been identified. However, the etiologic factors in Paget's disease remain elusive. A cause and effect relationship for the paramyxoviral infection and SQSTM1/p62 gene mutations responsible for pagetic osteoclast development and disease severity are unclear. This article will highlight the etiologic factors involved in the pathogenesis of Paget's disease.

Key words. Paget's disease; osteoclast; measles virus; sequestosome (p62); RANK ligand (RANKL).

Paget's disease of bone is a chronic focal skeletal disease that affects 2–3% of the population over the age of 60, with an increased incidence in Caucasians. The disease is associated with deformity and enlargement of single or multiple bones, among which the skull, clavicles, long bones and vertebral bodies are the most frequently involved [1]. Patients with Paget's disease are frequently asymptomatic, but approximately 10–15% have severe symptoms including bone pain, fractures, neurological complications due to spinal cord compression or nerve entrapment syndromes, deafness, and dental abnormalities. Paget's disease is a highly localized disease, and new lesions rarely develop during the course of the disease. It can be monostotic or polyostotic and the bone lesions continue to progress in size if untreated. Studies have also indicated that patients with Paget's disease have an increased incidence of osteosarcoma, approximately 1% of them developing osteosarcoma in an affected bone. Paget's disease has a very unusual geographic distribution, with an increased incidence in Caucasians of European descent, but it also occurs in African Americans. It is rare in those of Asian descent. Studies have also suggested high prevalence rates of radiographic Paget's disease in Britain, Australia, North America and western Europe. The incidence of Paget's disease appears to have been decreasing over the last several decades [2, 3], but the basis for this decrease is unknown.

Familial expansile osteolysis (FEO) is a rare disease related to Paget's disease, but occurs in patients at a much younger age and is a much more severe disease linked to activating mutations in the gene encoding the receptor activator of nuclear factor κB (RANK) on chromosome 18q [4].

Juvenile Paget's disease is characterized by widespread involvement of the skeleton, distinguishing it from Paget's disease of adults. It is caused by a homozygous deletion of the gene on chromosome 8q24.2 that encodes osteoprotegerin (OPG), a member of the tumor necrosis factor receptor family [5].

The primary pathologic abnormality in patients with Paget's disease is increased bone resorption, followed by abundant new bone formation that is disorganized and of

poor quality. Paget's disease has been described as a slow paramyxoviral infection process, suggesting a viral etiology for the disease. Familial incidence is common in Paget's disease and 40% of patients with the disease have an affected first-degree relative. Familial Paget's disease has an equal incidence in males and females. It is also evident that genetic factors play an important role in the familial and sporadic forms of Paget's disease of bone. Genetic linkage analysis has further indicated that Paget's disease is an autosomal dominant trait with genetic heterogeneity and incomplete penetrance. In this review, viral, genetic, and other etiologic factors that play an important role in the pathogenesis of Paget's disease of bone will be discussed.

Paramyxoviral etiology

A viral etiology has been proposed for Paget's disease due to an initial description of nucleocapsid-like structures in the nuclei and cytoplasm of pagetic osteoclasts by electron microscopy [6]. Immunocytochemical studies further confirmed that these nuclear inclusions cross-reacted with antibodies that recognized measles virus (MV) or respiratory syncytial virus (RSV) nucleocapsid antigens [7]. In situ hybridization techniques also identified the presence of MV messenger RNA sequences in up to 90% of osteoclasts and other mononuclear cells in pagetic bone specimens. Similarly, canine distemper virus (CDV) nucleocapsid antigens were also detected in osteoclasts from patients with Paget's disease [8]. These paramyxoviral-like nuclear inclusions are not unique to Paget's disease and were reported in patients with FEO and rarely in patients with osteopetrosis, pycnodysostosis, otosclerosis, and oxalosis [9]. This has raised the possibility that the virus may be a non-etiologic agent in a cell altered by a genetic defect. Alternatively, there may be sequence homologies between viral and cellular proteins. Since paramyxoviruses are RNA viruses, it is most unlikely that part of the viral genome is integrated into the genome of the affected population.

We have previously identified the expression of MV nucleocapsid (MVNP) transcripts in freshly isolated bone marrow cells from patients with Paget's disease. These MVNP transcripts contain mutations which resulted in amino acid substitutions clustered at the C-terminal end [10]. The mutations occured at a 1% rate in the total MVNP gene isolated from a patient with Paget's disease. We further demonstrated that osteoclast precursors, the granulocyte macrophage colony-forming unit (CFU-GM), as well as mature osteoclasts from patients with Paget's disease, expressed MVNP transcripts. We also detected expression of MVNP transcripts in peripheral blood-derived monocytes from these patients, indicating that MV infection occurs in early osteoclast lineage cells [11]. MV infection has a similar incidence worldwide and occurs in very young patients, whereas Paget's disease is a disease of the elderly. These observations suggest that if paramyxoviruses have an etiologic role in Paget's disease, these viral infections must persist for long periods of time. Pluripotent hematopoietic stem cells, which can persist for long periods of time in a quiescent phase, may be the initial target for the paramyxoviral infection in patients with Paget's disease. We found that other hematopoietic lineages from patients with Paget's disease in addition to the osteoclast lineage, including the erythroid and the erythroid precursors, burst-forming unit-erythroid (BFU-E), and multipotent myeloid precursors (CFUGEMM) also express MVNP transcripts [11]. Thus, if the initial site of infection occurs in a small number of primitive pluripotent hematopoietic stem cells that predominantly remain in Go, this might explain the chronicity of the infection. Also, there may be a genetic predisposition for chronic paramyxoviral infections of hematopoietic precursors in patients with Paget's disease. However, a cause and effect relationship of paramyxoviruses in Paget's disease remains to be proven, as no infectious virus has been isolated from pagetic cells. Also, it is not clear how the focal lesions are initiated in Paget's disease. In contrast to these results, other workers have been unable to detect paramyxoviral nucleocapsid transcripts in samples obtained from patients with Paget's disease [12, 13].

The presence of paramyxoviral transcripts in osteoclasts and osteoclast precursors from patients with Paget's disease suggests a pathophysiologic role for the viral genes in the development of the pagetic lesions. In studies using normal osteoclast precursors (CFU-GM) transduced with retroviral vectors expressing the MVNP gene, the cells formed pagetic-like osteoclasts more rapidly, with an increased number of nuclei, hypersensitivity to 1,25-dihydroxyvitamin D3 (1,25-$[OH]_2D_3$), and an increased bone-resorbing capacity compared to normal osteoclasts. In contrast, normal osteoclast precursors transduced with the MV matrix gene did not express an abnormal phenotype [14]. Furthermore, infecting canine bone marrow cells with CDV results in the development of multinucleated cells that share some of the phenotypic characteristics of pagetic osteoclasts [15]. More recently, CDV was shown to be infectious to human osteoclast precursors and to enhance osteoclast differentiation and function. Previously, we have targeted CD46, the human MV receptor, to cells of the osteoclast lineage in transgenic mice and demonstrated that MV infection of osteoclast precursors from CD46 transgenic mice form osteoclasts, which express a pagetic phenotype *in vitro* [16]. However, TRAP-CD46 mice do not develop sustained MV infection, most likely reflecting the need for blocking interferon production for development of persistent MV infection in these mice. Transgenic mice targeted with

MVNP expression to cells of the osteoclast lineage in vivo results in a bone phenotype that is characteristic of Paget's disease and supports a pathophysiologic role for MVNP in Paget's disease. However, these studies do not exclude genetic factor(s) that may play an important role in disease severity and pathogenesis.

Taken together, these data suggest a potential pathophysiologic role for the paramyxoviral nucleocapsid gene that is expressed in patients with Paget's disease. Mouse models of MV infection were also developed in which CD46 is introduced into transgenic mice and bred to another transgenic mouse lacking the alpha-beta interferon receptor. Upon exposure to MV, these mice developed immune suppression similar to patients with acute MV infection. The mice lacking the alpha-beta interferon receptor demonstrated persistence of MV infection for at least 12 days [17]. Although several lines of evidence support a viral etiology for Paget's disease, it is still unclear how this is related to the late onset and focal nature of Paget's disease.

Genetic linkage of sequestosome 1/p62 and molecular signaling

A genome-wide search in familial Paget's disease of bone indicated genetic heterogeneity of the disease, with candidate loci on chromosomes 2q, 5q, 6p, 10p, and 18q [18, 19, 20]. Linkage studies, coupled with mutation screening, have excluded involvement of RANK and also osteoprotegerin in the majority of patients with Paget's disease of bone [21]. Genetic studies have demonstrated linkage in 7 of 7 patients with osteosarcoma to loss of heterozygosity in a region of 18q that is adjacent to or within a locus for Paget's disease on 18q [22].

Recently, the sequestosome 1 gene encoding the protein p62 (SQSTM1/p62) mapped within the critical region on chromosome 5q35-qter identified a proline-leucine amino acid change at codon 392 (P392L) in French-Canadian patients with Paget's disease of bone [23]. The frequency of the mutation was 16% and 46% for sporadic and familial cases tested, respectively. Further studies also identified different mutations affecting the highly conserved ubiquitin-associated (UBA) domain of the SQSTM1/p62 protein in patients with familial and sporadic Paget's disease [24–26]. In addition to the P392L mutation, two novel mutations (M404V and G425R) were also identified in exon 8 of the SQSTM1 gene in Italian sporadic patients; however, no significant differences in the clinical history was observed in these patients [27]. Studies with patients with familial disease in The Netherlands further identified three new mutations, S399P, M404T, and G425R, which correlated with serum alkaline phosphatase activity similar to patients with P392L mutations [28]. Insertion mutations introducing a stop codon or abolishing the splice donor site at the start of the intron 7 region of SQSTM1 were also identified in UK-derived familial and sporadic Paget's disease cases [29].

Structural analysis studies have classified p62 mutations that retain or abolish the ability of the isolated UBA domain to bind to K48-linked polyubiquitin [30]. Cavey et al. [31] have recently studied the effects of various p62 mutants associated with Paget's disease on the *in vitro* ubiquitin-binding properties of p62 protein. These studies indicated that several SQSTM1 mutations associated with Paget's disease impair p62 binding to ubiquitinylated targets at physiological temperature, suggesting that p62 mutations predispose to Paget's disease through a common mechanism that depends on loss of ubiquitin binding by p62. Using an *in vitro* expression cloning approach, 11 proteins that interact with the p62 UBA domain that are associated with neurodegenerative disorders have been identified [32]. These studies have shown that the heat shock protein-70 (HSP70) interacts with the p62 UBA domain. HSPs are well known to play a role in protein binding, assembly, intracellular transport and degradation within cells. Development of methods such as the yeast two-hybrid screening of an osteoclast cDNA library using the p62 UBA domain as bait should identify further genes that play an important role in pagetic osteoclast development.

The atypical protein kinase C (aPKC) interaction with SQSTM1/p62 has been implicated in signaling cascades that control NF-κB activation (fig. 1). It is evident that p62 provides a scaffold linking the aPKCs to the tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1) receptor signaling complexes through its interaction with RIP and TRAF-6, respectively [33]. Thus, SQSTM1/p62 mediates IL-1 and TNF-α cytokine signaling to activate NF-κB. TRAF-6, plays an essential role in receptor activator of NF-κB ligand (RANKL) signaling during osteoclastogenesis. Recently, RANKL stimulation has been shown to result in upregulation of p62 expression in osteoclast precursor cells, and the genetic inactivation of p62 in mice impaired PTHrP-induced osteoclastogenesis *in vivo*. However, p62 null mice have a grossly normal skeletal phenotype and no alterations were found in the trabecular size and number of osteoclasts compared to wild type mice. In vitro studies demonstrated that p62 deficiency leads to inhibition of IKK activation and NF-κB nuclear translocation during osteoclastogenesis [34]. These studies also demonstrated that RANKL stimulation induces formation of a ternary complex involving TRAF-6, p62 and aPKC during osteoclastogenesis. Recent evidence indicates that TNF-α stimulation of osteoclast precursors in the presence of cofactors such as transforming growth factor-beta (TGF-β) results in osteoclastogenesis independent of the RANKL-RANK-TRAF-6 axis [35]. Also, TRAF-2 has been shown to be essential for TNF-α signaling to induce osteoclastogenesis [36]. It is unclear if p62

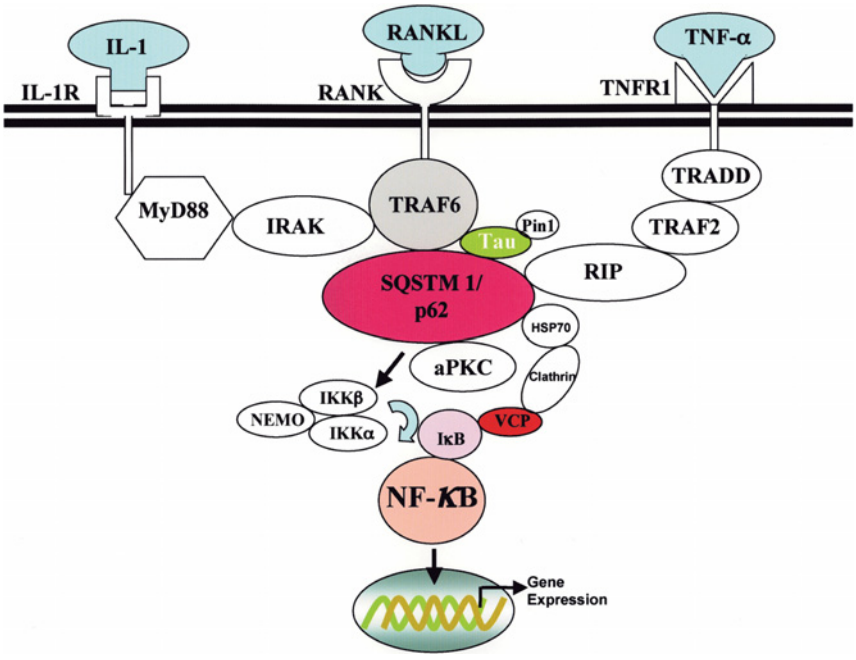

Figure 1. Sequestosome 1 (SQSTM1/p62) protein-protein interactions and associated cellular signaling cascades. RANKL-RANK signaling induces p62 to form a ternary complex with TRAF-6 and aPKCs during osteoclast differentiation. Several mutations in the UBA domain of SQSTM1/p62 have been identified in patients with Paget's disease. Similarly, mutations in VCP, a multiubiquitin chain-targeting factor required in ubiquitin-proteasome degradation have been shown to cause inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia.

UBA mutants affect the status of NF-κB activation and result in a pagetic phenotype of the osteoclast. However, it is reasonable to assess ligand specificity and alternative signaling mechanisms or alternative protein-protein interactions in pagetic-like osteoclast development. For example, it has been shown that inositol 5′ phosphatase-deficient mice are severely osteoporotic with an increased number of osteoclast precursors and hyperactive osteoclasts. In addition, serum levels of IL-6 are markedly increased in these mice as in Paget's disease [37].

p62 was shown to bind ubiquitin non-covalently and sequester into cytoplasmic aggregates in some systems. p62 has been proposed to function as a polyubiquitin shuttling factor for proteasomal degradation through its interaction with the proteasome. Therefore, it is reasonable to speculate that the occurrence of mutations in the UBA domain of p62 in patients with Paget's disease results in cellular accumulation of insoluble polyubiquinated protein aggregates due to failure of proteasomal degradation. For example, accumulation of hyperphosphorylated Tau, the microtubule-associated protein in the brain of patients with Alzheimer's disease, contributes to neurodegeneration. Recently Tau has been identified as a K63-polyubiquitinated substrate of TRAF6 that interacts with the UBA domain of p62 and is targeted for proteasomal degradation [38]. Furthermore, recent evidence also suggests that ubiquitin-proteasome regulatory mechanisms play an important role in osteoblast differentiation [39]. Smad ubiquitin regulatory factor-1 (Smurf1) ubiquitin ligase deficiency results in an age-dependent increase in bone mass due to accumulation of phosphorylated MEKK2 and activation of the JNK signaling cascade in osteoblast cells [40]. Although patients with Paget's disease demonstrate high levels of alkaline phosphatase activity, the molecular defect or alteration in osteoblast cells and the role that the p62 UBA mutant may play in osteoblast activity in these patients is not clear. Osteoblasts are also increased in lesions in patients with Paget's disease, and they appear to be morphologically normal.

Normal human osteoclast precursors transduced with a P392L mutant p62 retroviral expression vector displayed enhanced sensitivity to RANKL and increased osteoclast formation. However, the osteclast precursors demonstrated no pagetic characteristics such as hypersensitivity to 1,25-$(OH)_2D_3$ and increased number of nuclei in the osteoclasts formed *in vivo*. Furthermore, transgenic mice with the P392L mutant p62 gene targeted to cells in the osteoclast lineage using the tartrate-resistant acid phosphatase (TRAP) promoter demonstrated increased osteoclast numbers and were osteopenic but did not develop the increased osteoblast activity that is characteristic of pagetic lesions. These studies suggested that the P392L mutation in p62 enhances osteoclast formation, possibly through increased RANK signaling. Therefore, the precise role that SQSTM1/p62 and signaling mechanisms

may play in pagetic-like osteoclast development and pathogenesis of disease remain to be elucidated.

Inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia (IBMPFD) were recently reported to be caused by mutant valosin-containing protein (VCP) that maps to chromosome 9p21.1-p12 [41]. VCP, a member of the AAA-ATPase superfamily, is a multiubiquitin chain targeting factor for proteasome degradation. VCP is known to function in cell cycle control, membrane fusion, and the ubiquitin-proteasome degradation pathway. It has also been shown that VCP may provide a physical and functional link between IKB-α and the 26S proteasome and play an important role in the proteasome degradation of IKB-α [42, 43]. Formation of protein aggregates and or accumulation of cellular signaling molecules may occur upon failure of proteasomal degradation due to mutations in the N-terminal ubiquitin-binding domain of VCP or UBA domain of p62. This ubiquitous mechanism does not explain specific molecules associated with the pathogenesis of Paget's disease or dominant negative effects on gene expression which regulate osteoclastogenesis and bone resorption activity. However, identification of molecules which interact with p62/VCP provide further insights into ligand specificity for altered signaling cascades responsible for pagetic-like osteoclast development. Recent evidence suggests that a fraction of IKB-α physically associates with nuclear corepressors and histone acetylases. It has further been shown that recruitment of IKKs to the nucleus in response to TNF-α may induce chromatin-associated IKB-α release and gene activation [44]. However, genetic linkage analysis indicated that mutations in the p62 gene may not completely account for the pathogenesis of Paget's disease. The severity of disease in family members carrying the same mutation can vary widely, and up to 20% of individuals who harbor p62 mutations and are older than 55 years do not have PD. Therefore, it is reasonable to hypothesize that other gene loci may be involved in the genetic predisposition and osteoclast abnormalities associated with Paget's disease of bone.

Gene polymorphisms and mRNA expression in Paget's disease

Several studies have examined the linkage of HLA begenes cause of their highly polymorphic nature, and significant associations were observed between class II antigens and Paget's disease [45]. Similarly, studies also indicated that the TNFRSF11B gene encoding OPG with lysine at the codon 3 position predisposes to the development of sporadic and familial forms of Paget's disease that is not caused by SQSTM1 mutations [46]. Recent studies also identified significant variations in genotype frequency of polymorphisms in estrogen receptor-alpha and calcium-sensing receptor genes in patients with Paget's disease compared to normal subjects, which may contribute genetic susceptibility to Paget's disease [47]. Allelic association determines the risk of disease severity and susceptibility but does not explain the focal nature of the disease. Inhibition of apoptosis has been hypothesized to lead to an increased osteoclast lifespan resulting in an increase in the size and number of osteoclasts responsible for enhanced bone resorption activity in patients with Paget's disease. In support of this, in situ hybridization studies have identified increased levels of Bcl2 mRNA expression in pagetic osteoclasts. Further studies indicated that the polymorphic mutations present in the Bcl2 gene promoter region are responsible for elevated Bcl2 expresssion in patients with paget's disease [48]. In situ hybridization studies have also identified increased levels of IL-6 and c-fos proto-oncogene mRNA expression in pagetic osteoclasts. IL-6 receptor and NF-IL-6 mRNA levels were also increased in osteoclasts from bone samples from patients with Paget's disease compared to those with osteoarthritis [49]. It is essential to delineate whether enhanced gene expression or half-life of mRNA and recruitment of coactivators for gene transcription play a pivotal role in pagetic osteoclast development. This is evident from the studies which indicated that pagetic osteoclast precursors are hypersensitive to 1,25-$(OH)_2D_3$ compared to normals. The increased sensitivity of osteoclast precursors from Paget's patients to 1,25-$(OH)_2D_3$ is mediated through the vitamin D3 receptor (VDR); however, this is not due to increased numbers of VDR in pagetic osteoclast precursors compared to normals, but appears to be due to enhanced affinity of the VDR in pagetic cells for its ligand compared to normals [50]. In support of a viral etiology, MVNP gene expression in osteoclast precursors was recently demonstrated to result in increased levels of TAF_{II}-17 transcription factor gene expression. The high levels of TAF_{II}-17 permit formation of a VDR transcription complex at low levels of receptor occupancy by 1,25-$(OH)_2D_3$ [51]. These results support the hypothesis that part of the pathophysiology underlying the increased osteoclast activity in Paget's disease is due to increased levels of VDR coactivators that enhance VDR-mediated gene transcription at low levels of 1,25-$(OH)_2D_3$. Enhanced levels of general transcription factors such as TAF_{II}-17 may not explain the cellular specificity and chronic pathogenesis. However, this does not exclude the possibility of their involvement in pagetic osteoclast development at the involved sites.

Systemic factors

Bones not clinically involved with Paget's disease appear to show increased bone remodeling. This increased bone remodeling in unaffected bones has been ascribed to sec-

ondary hyperparathyroidism rather than to subclinical involvement of the bones with Paget's disease. However, less than 20% of patients with Paget's disease have elevated parathyroid hormone (PTH) levels [2]. Enhanced levels of IL-6, RANKL, M-CSF and endothelin-1 have been associated with Paget's disease. These systemic factors are implicated in the pathogenesis and as an indicator of disease activity [52, 53]. We have recently detected elevated levels of high molecular-weight serum kininogen in patients with Paget's disease [unpublished data]. Because Paget's lesions are focal, pagetic cells may be more sensitive to the elevated systemic factors. The increased levels of IL-6 in the peripheral blood of patients with Paget's disease may in part explain the increased bone remodeling seen in bones not clinically involved with Paget's disease. We need, therefore, to define a pathologic role of systemic factors that are upregulated in patients with Paget's disease. Identification of such factors will also provide further insights into the localized nature and progression of pagetic lesions and disease activity. Osteoclasts from patients with Paget's disease also appear to produce increased levels of IL-6 and express higher levels of IL-6 receptors than normal osteoclasts. IL-6, which is a stimulator of human osteoclast formation, may act as an autocrine/paracrine factor to enhance osteoclast formation in patients with Paget's disease and increase the osteoclast precursor pool. The number of early osteoclast precursors, CFU-GM, was increased significantly in marrow aspirates from patients with Paget's disease compared to normals [54]. The osteoclast precursors from patients with Paget's disease also appear to be hyperresponsive to RANKL and marrow stromal cells from pagetic lesions have increased RANKL expression [55, 56]. RANKL is a critical osteoclast differentiation factor that is expressed on marrow stromal and osteoblast cells in response to several osteotropic factors. The increased sensitivity of osteoclast precursors from Paget's patients to RANKL appears to be due to interactions of these precursors with IL-6. Therefore, it has been hypothesized that Pagetic osteoclasts expressing the MVNP gene produce high levels of cytokines that increase the osteoclast precursor pool. Chronic exposure to cytokines produced by the pagetic osteoclasts results in constitutive overexpression of RANKL in stromal/osteoblast cells further enhancing the abnormal osteoclast development and localized nature in pagetic bone lesions in patients with Paget's disease [57]. Although osteoclasts are thought to be the primary cells affected in Paget's disease, osteogenic cells may be either indirectly or directly affected by the elevated systemic factors or intrinsic genetic defect. Immature osteoblasts are the major responders to RANKL-inducing cytokines and studies have also suggested that expression of RANKL decreases with osteoblast maturation [58]. Therefore, the increased numbers of highly active osteoblasts rapidly form large amounts of woven bone in patients with Paget's disease.

Conclusions and perspectives

In recent years significant progress has been made with respect to etiologic factors associated with Paget's disease of bone, an autosomal dominate trait with genetic heterogeneity. Although recurrent mutations in the UBA domain of sequestosome 1 (SQSTM1/p62) in patients with Paget's disease have been identified and implicated as a common cause of familial and sporadic Paget's disease, it is still unclear if mutant p62 is sufficient to cause Paget's disease, and what its precise role is in osteoclast abnormalities. Future perspectives are to identify novel cellular protein interactions with the ubiquitin-binding domain of VCP or the UBA domain of p62 and to develop animal models to further delineate the role of SQSTM1/p62-associated signaling cascades in pagetic osteoclast development. Lack of skeletal abnormalities in p62-deficient mice further suggests a potential role for genes present in other candidate loci that have been linked with Paget's disease. Alternatively, a genetic defect may favor environmental factors such that MV infection plays a potential role in the pathogenesis of the disease. However, the molecular basis for the abnormalities associated with osteoclasts, the role of paramyxoviral infection, and the persistence of the virus in patients with Paget's disease are unclear. It will be important to determine a cause and effect relationship for the persistence of paramyxoviral infection and genetic predisposition in patients with Paget's disease. Essential also, will be to define the role of elevated systemic factors and underlying molecular mechanisms in the initiation and progression of focal lesions in patients with Paget's disease.

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Elevated Serum Kininogen in Patients with Paget's Disease of Bone: A Role in Marrow Stromal/Preosteoblast Cell Proliferation

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Abstract Paget's disease (PD) of bone is a chronic focal skeletal disorder characterized by excessive bone resorption followed by abundant new bone formation. Enhanced levels of IL-6, RANKL, M-CSF, and endothelin-1 have been associated with PD. In the present study, we identified increased serum levels (2 to 5-fold) of inflammatory cytokine, kininogen (KNG) in patients with PD compared to normal subjects. Treatment of pagetic bone marrow derived stromal/preosteoblast cells with recombinant KNG (25 ng/ml) for 24 h period resulted in a 5-fold increase in the levels of phospho-HSP27 and a 3-fold increase in ERK1/2 phosphorylation in these cells. However, pagetic stromal cells stimulated with KNG in the presence of ERK activation inhibitor peptide did not significantly affect the levels of phospho-HSP27. KNG increased normal and pagetic marrow stromal cell proliferation at 1.4-fold and 2.5-fold, respectively. KNG in the presence of an ERK inhibitor peptide did not stimulate pagetic marrow stromal cell proliferation. Furthermore, siRNA suppression of HSP27 expression significantly decreased KNG inhibition of etoposide-induced caspase-3 activation and apoptosis in these cells. In summary, KNG modulate bone marrow derived stromal/preosteoblast cell proliferation and suppress etoposide-induced apoptosis through ERK and HSP27 activation, respectively. These results implicate a pathophysiologic role for KNG in patients with PD. J. Cell. Biochem. 98: 1681–1688, 2006.

Key words: kininogen (KNG); Paget's disease; stromal/preosteoblast cells; extracellular signal-regulated kinase (ERK); heat-shock protein

Paget's disease (PD) of bone is a chronic focal skeletal disorder that affects up to 2%–3% of the population over the age of 60 years. The pathologic abnormality in patients with PD involves increased bone resorption by the osteoclasts, followed by abundant new bone formation that is of poor quality [Roodman and Windle, 2005]. Genetic linkage analysis indicated that 40% of patients with PD have an affected first degree relative and 1% of patients develop osteosarcoma [Hansen et al., 1999]. PD is an autosomal dominant trait with genetic heterogeneity. Recurrent mutations in the ubiquitin-associated domain of Sequestosome 1 (SQSTM1/p62) are identified in patients with PD [Laurin et al., 2002; Johnson-Pais et al., 2003; Hocking et al., 2004]. Osteoclasts and osteoclast precursors from patients with PD contain paramyxoviral transcripts and appear hyperresponsive to 1,25-$(OH)_2D_3$ and RANK ligand (RANKL) [Neale et al., 2000; Roodman and Windle, 2005]. However, a cause and effect relationship for the paramyxoviral infection and SQSTM1/p62 gene mutations associated with this disease and osteoclast abnormalities are unclear.

The biochemical markers provide an integrated assessment of the cellular events occurring throughout the skeleton of patients with PD. Interleukin-6 (IL-6) levels were shown to increase in bone marrow plasma and peripheral blood of patients with PD. These studies further indicated that IL-6 is an autocrine/paracrine factor, which stimulates human osteoclast formation and increase the osteoclast precursor

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pool in patients with PD. In addition, RANKL, a critical osteoclastogenic factor that is expressed on marrow stromal/osteoblast cells is also upregulated in areas involved with PD. The increased sensitivity of osteoclast precursors from Paget's patients to RANKL appears to be due to interactions of these precursors with IL-6. Addition of neutralizing antibodies to IL-6 decreased the RANKL sensitivity of osteoclast precursors to normal levels. Similarly, addition of IL-6 to cultures of normal osteoclast precursors enhanced the responsivity of these precursors to RANKL to the levels seen with pagetic osteoclast precursors. The enhanced expression of RANKL and IL-6 in pagetic lesions could contribute to the abnormal osteoclast development and highly localized nature of PD [Roodman and Windle, 2005]. In situ hybridization studies have further identified increased levels of IL-6, c-fos proto-oncogene, Bcl-2 anti-apoptotic gene mRNA expression in pagetic osteoclasts [Hoyland et al., 1994; Brandwood et al., 2003].

Urinary *N*-telopeptide, pyridinoline, and deoxypyridinoline have all been reported to be more specific indices of skeletal matrix resorption and are not influenced by dietary gelatin. Furthermore, serum calcium levels are typically normal in PD and also serum osteocalcin levels appear to be a poor index of the progression of the disease. The increased bone remodeling in unaffected bones has been ascribed to secondary hyperparathyroidism rather than to subclinical involvement of the bones with PD. However, less than 20% of patients with PD have elevated parathyroid hormone (PTH) levels [Siris, 1998]. Serum tartrate resistant acid phosphatase (TRAP), presumably released by osteoclasts, appears to be an index of bone resorption in PD but is not routinely used. The most useful markers for the increased osteoblast activity in PD are the total alkaline phosphatase and bone-specific alkaline phosphatase activity levels in serum [Reddy, 2004]. It has been reported that serum M-CSF levels are significantly elevated in patients with PD, however not significantly different in patients under treatment compared to normal subjects [Neale et al., 2002]. Patients also showed significantly higher endothelin-1 circulating levels than controls with a positive correlation with serum alkaline phosphatase, but not with urinary hydroxyproline [Tarquini et al., 1998]. In the present study, we identified high-level expression of an inflammatory cytokine, kininogen (KNG) in patient's sera compared to normal subjects, and further demonstrated potential role that KNG may play in marrow stromal cell proliferation. Our results further implicate a pathophysiologic role for KNG in PD.

MATERIALS AND METHODS

Materials

Recombinant human KNG was purchased from R&D systems, Inc. (Minneapolis, MN). Extracellular signal-regulated kinase (ERK) activation inhibitor peptide I was obtained from Calbiochem (Darmstadt, Germany).

Western Blot Analysis

Normal and pagetic bone marrow derived stromal/preosteoblast cells were isolated as described previously [Roccisana et al., 2004]. The cells were seeded in six-well plates at a density of 10^6 cells in 10 ml of α-minimal essential medium (MEM) containing 10% fetal calf serum (FCS) and cultured for 24 h in the presence of human recombinant KNG. The cells were lysed in a buffer containing 20 mM Tris, pH 7.4, NaCl 150 mM, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 200 μM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and aprotinin (1 mg/ml). The protein content of the samples was measured using the BCA method as per the manufacturer's protocol (Pierce, Rockford, IL). Serum (4 μg total protein) or cell lysates (15 μg protein) samples were then subjected to SDS–polyacrylamide gel electrophoresis (PAGE), using 12% Tris-HCl gels. The proteins were transferred from SDS gels onto a PVDF membrane (Bio-Rad Laboratories, Hercules, CA) for immunoblot analysis. Blocking was performed with 5% non-fat dry milk in 150 mM NaCl, 50 mM Tris, pH 7.2, 0.05% Tween-20 (TBST) buffer. The membrane was then incubated for 1 h with anti-KNG antibody (The Binding Site, Birmingham, UK), and anti-ERK1/2, phospho-ERK1/2, HSF-2 (heat-shock factor-2), RANKL mouse monoclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti-HSP27 (heat-shock protein-27), phospho-HSP27 (P-HSP27) antibodies (Cell signaling technology, Inc., Beverly, MA) diluted 1:500 in 5% non-fat dry milk-TBST. The blots then were incubated for 1 h with horseradish

peroxidase-conjugated goat anti-mouse IgG, diluted 1:2,500 in 5% non-fat dry milk-TBST, and developed using an ECL system (Amersham Biosciences, Little Chalfont, UK). NIH image program (National Institutes of Health, Bethesda, MD) was used for quantification analysis after digital scanning of the exposed X-ray films.

Inhibition of ERK Activation

Inhibition of ERK activation was performed as described previously [Kelemen et al., 2002]. Briefly, serum-starved pagetic bone marrow stromal/preosteoblast cells were treated with the ERK activation inhibitor peptide (25 μM) for 4 h at 37°C and then stimulated with KNG (25 ng/ml) for an additional 24 h in α-MEM containing 10% FCS. Cells were washed with ice-cold phosphate buffered saline, suspended in 0.5 ml of icecold lysis buffer, and scraped from flasks. Cell lysate was sonicated, centrifuged to remove remaining insoluble material, and measured protein concentration. The protein concentration in the cell lysate was determined by BCA protein assay system (Pierce Chemical Co.).

Cell Proliferation Assay

Normal and pagetic human bone marrow stromal/preosteoblast cell proliferation was determined using a CellTiter 96 non-radioactive cell proliferation assay kit (Promega, Madison, WI). The cells were seeded at 5×10^3 cells/well on 96-well plates, and incubated in the presence or absence of KNG (0–50 ng/ml) for 48 h at 37°C in humidified, 5% CO_2 atmosphere. After 48 h period, cell proliferation rate was assayed following the manufacturer's protocol.

To determine the role of HSP27 in cell proliferation, we have used siRNA to suppress HSP27 expression in pagetic stromal cells. The cells were seeded at 5×10^3 cells/well in a 96-well plate and transiently transfected with HSP27 siRNA or control siRNA (10 nM) (Santa Cruz Biotechnology, Inc.) by Lipofectamine method. Briefly, 1 μl of 10 μM siRNA, 100 μl of α-MEM media, and 6 μl of Lipofect AMINE Plus reagent (Invitrogen, Grand Island, NY) were premixed for 15 min at room temperature. During this time, 5 μl of Lipofect AMINE transfection reagent was mixed with 100 μl of α-MEM media. The two mixtures were then combined and incubated for 15 min at room temperature to form a complex. The reaction mixture was diluted with 800 μl of α-MEM medium and 100 μl aliquot of the entire mixture was added to each well. After 12 h, the cells were treated with α-MEM containing 10% FCS with KNG (0.5, 25 ng/ml) for additional 48 h, and subjected to the proliferation assay.

Inhibition of ERK phosphorylation was performed by seeding the pagetic stromal cells at 5×10^3 cells/well in 96-well plates, and treated with the ERK activation inhibitor peptide (25 μM) for 4 h. The cells were then stimulated with KNG (0–25 ng/ml) for an additional 48 h in α-MEM containing 10% FCS, and subjected to the proliferation assay. Each treatment was analyzed in triplicate and the results represent mean values of three independent experiments ($P < 0.05$).

Apoptosis Assay

To determine the effect of KNG and role of HSP27 in stromal/preosteoblast cell apoptosis, Paget's bone marrow stromal cells were transiently transfected with HSP27 siRNA or control siRNA as described above. The cells were stimulated with KNG (25 μM) for 24 h and cultured in the presence of 25 μM etoposide (Sigma-Aldrich, St. Louis, MO) at 37°C for additional 24 h period. Total cell lysates were prepared with a lysis buffer containing 25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin. The supernatant of the cell lysate (100 μg of protein) was then assayed for caspase-3 activation to detect cell apoptosis using the CaspACE assay system (Promega) following the manufacturer's protocol.

Etoposide-induced apoptosis in pagetic stromal/preosteoblast cells was confirmed by immuno-fluorescence method. Briefly, paget's bone marrow stromal cells were cultured on glass coverslips in the presence of KNG (25 ng/ml) or etoposide (25 μM) alone, and in combination for 4 h. The cells were fixed in 4% paraformaldehydephosphate buffered saline buffer and permeabilized with 0.1% Triton X-100 in PBS. The cells were incubated overnight with a mouse anti-cytochrome c antibody (PharMingen, San Diego, CA) diluted 1:1,000 in PBS containing 1% bovine serum albumin. We used an Alexa ($\lambda_{ex} = 488$ nm and $\lambda_{em} = 519$ nm)-coupled anti-mouse IgG (1:600 dilution) to detect cytochrome c release using a confocal microscope.

RESULTS

Identification of KNG Overexpression in the Serum from Patients with PD

Enhanced levels of KNG (63 kDa) are associated with inflammatory conditions [Colman and Schmaier, 1997; Carretero, 2005]. In the present study, we examined KNG expression in serum samples obtained from normal subjects and patients with PD by Western blot analysis. As shown in Figure 1, Western blot analysis of serum samples (4 μg total protein) of five representative individuals from a total of nine PD patients and normals analyzed further indicated 2 to 5-fold increases in levels of KNG (63 kDa) in patients with PD compared to normal subjects. These data are consistent with a potential pathologic role for KNG in PD.

KNG Enhances ERK and HSP27 Phosphorylation in Pagetic Marrow Stromal/Preosteoblast Cells

RANKL, a critical osteoclastogenic factor that is expressed on marrow stromal/osteoblast cells is upregulated in PD. We have recently demonstrated that HSF-2 is a downstream target of b-FGF-induced RANKL expression in SAKA-T normal human bone marrow derived stromal cells [Roccisana et al., 2004]. We therefore, further examined the effects of KNG on HSP27 and ERK phosphorylation in pagetic marrow stromal cells. As shown in Figure 2, Western blot analysis of total cell lysates obtained from the pagetic stromal cells treated with KNG (25 ng/ml) for a period of 24 h demonstrated a significant increase (5-fold) in the levels of HSP27 phosphorylation compared to untreated cells. However, there was no significant change in the levels of HSF-2 expression in these cells. In addition, KNG enhanced a 3-fold increase in ERK1/2 phosphorylation compared with untreated cells (Fig. 3A). To further delineate if ERK signaling is involved in HSP27 phosphorylation, we used ERK1/2 activation inhibitor. Pagetic stromal cells were stimulated with

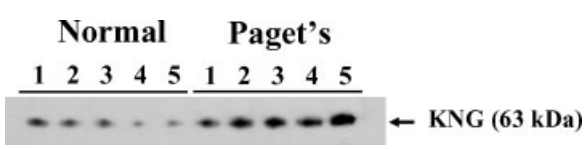

Fig. 1. Western blot analysis for KNG expression in normal and Paget's patient's serum. Serum samples (4 μg) from normal and Paget's patients shown are representative of total nine subjects of each analyzed. Sample loading was normalized for protein concentration.

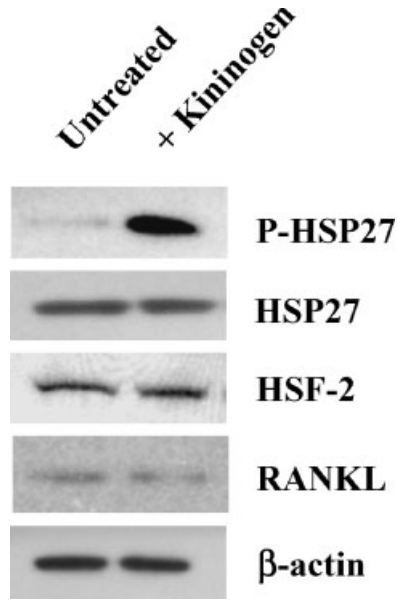

Fig. 2. KNG induces HSP27 phosphorylation. Pagetic marrow stromal/preosteoblast cells were treated with KNG (25 ng/ml) for 24 h, and total cell lysates (15 μg) were subjected to Western blot analysis using anti-human HSP27, P-HSP27, HSF-2, and RANKL antibodies as described in methods.

KNG in the presence of ERK activation inhibitor peptide-1 (25 μM), which binds to ERK2 and prevents interaction with MEK (mitogen-activated protein kinase kinase). Western blot analysis of total cell lysates obtained from

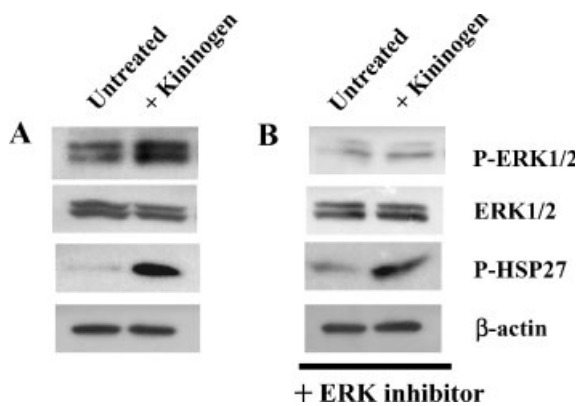

Fig. 3. KNG promotes ERK phosphorylation and ERK activation inhibitor did not affect HSP27 phosphorylation. **A**: KNG enhanced ERK and HSP27 phosphorylation. Pagetic stromal cells were stimulated with KNG (25 ng/ml) for 24 h, and total cell lysates (15 μg) prepared were subjected to Western blot analysis using anti-human ERK1/2, phospho-ERK1/2, and phospho-HSP27 as described. **B**: HSP27 phosphorylation is independent of ERK1/2 signaling pathway. Serum-starved pagetic stromal cells were treated with the ERK activation inhibitor peptide (25 μM) for 4 h at 37°C and then stimulated with KNG (25 ng/ml) for an additional 24 h. Total cell lysates (15 μg) obtained were subjected to Western blot analysis.

KNG stimulated cells did not demonstrate a significant change in the levels of phospho-HSP27 (Fig. 3B), suggesting that HSP27 phosphorylation is independent of ERK signaling pathway. We have observed similar effect of KNG using normal human bone marrow derived stromal cells (data not shown).

KNG Enhances Proliferation of Paget's Bone Marrow Stromal Cells Through ERK Activation

Since ERK signaling is associated with cellular proliferation, we have examined the potential of KNG to stimulate normal and pagetic bone marrow stromal/preosteoblastic cell growth. As shown in Figure 4, KNG treatment significantly increased normal and pagetic bone marrow stromal cells proliferation in a dose-dependent manner. KNG (25 ng/ml) increased normal and pagetic marrow derived stromal cell proliferation at 1.4-fold and 2.5-fold compared to untreated control cells, respectively. Since KNG significantly enhanced HSP27 and ERK phosphorylation in pagetic bone marrow derived stromal cells, we further examined if HSP27 and ERK signaling is involved in KNG stimulation of cell proliferation. We used siRNA and ERK inhibitor peptide

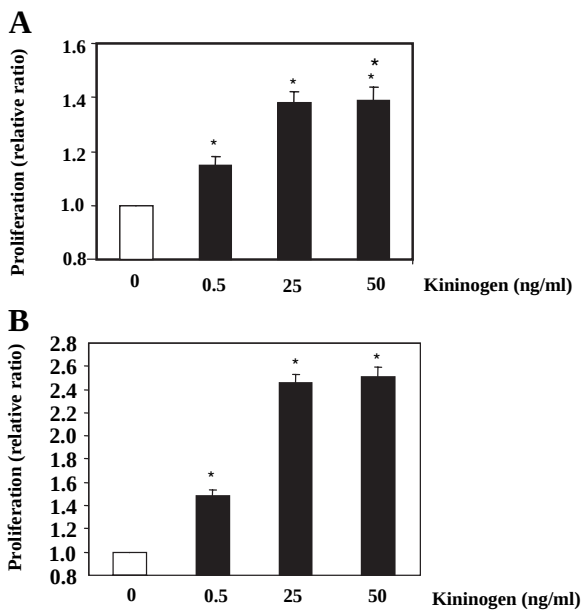

Fig. 4. Effect of KNG on proliferation of bone marrow stromal/preosteoblast cells. **A**: Normal human bone marrow derived stromal cells and (**B**) Pagetic marrow derived stromal/preosteoblastic cells were seeded at 5×10^3 cells/well in 96-well plates, and incubated in the presence or absence of KNG (0.5–50 ng/ml) for 48 h, and proliferation assay was performed as described in methods.

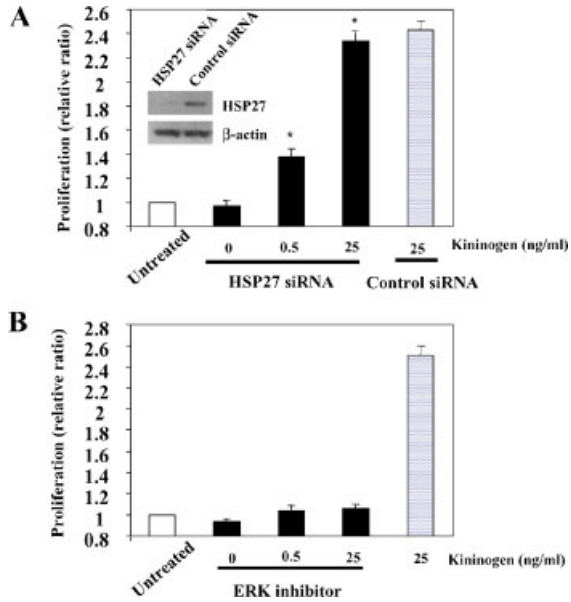

Fig. 5. KNG stimulate pagetic stromal cell proliferation through ERK signaling pathway. **A**: siRNA suppression of HSP27 expression did not affect KNG stimulated proliferation of pagetic marrow stromal cells. HSP27 or control siRNA (10 nM) was transiently transfected into pagetic stromal cells, and examined cell proliferation. Western blot analysis confirms the suppression of HSP27 expression (Inset). **B**: ERK activation inhibitor peptide suppressed the KNG stimulated proliferation of pagetic stromal cells. Pagetic marrow stromal cells were treated with ERK activation inhibitor peptide (25 μM) for 4 h at 37°C, and then stimulated with KNG (0.5, 25 ng/ml) for an additional 48 h and assayed for cell proliferation as described ($P < 0.05$).

to block the expression of HSP27 and ERK activation in these cells, respectively. As shown in Figure 5A, siRNA suppression of HSP27 expression did not affect the KNG stimulated proliferation of these cells. However, inhibition of ERK phosphorylation completely abolished the KNG stimulated cell proliferation (Fig. 5B). These results suggest that KNG enhances proliferation of Paget's bone marrow stromal/preosteoblast cells through ERK signaling pathway.

KNG Inhibits Etoposide-Induced Apoptosis in Paget's Bone Marrow Stromal Cells

It has been reported that HSP27 inhibits cellular apoptosis by preventing cytochrome c-triggered caspase-3 activation [Garrido et al., 1999]. Therefore, we further examined the effect of KNG on etoposisde-induced apoptosis and the role of HSP27 in Paget's bone marrow derived stromal/preosteoblast cells. Paget's stromal cells were transiently transfected with HSP27 siRNA and treated with KNG (25 ng/ml)

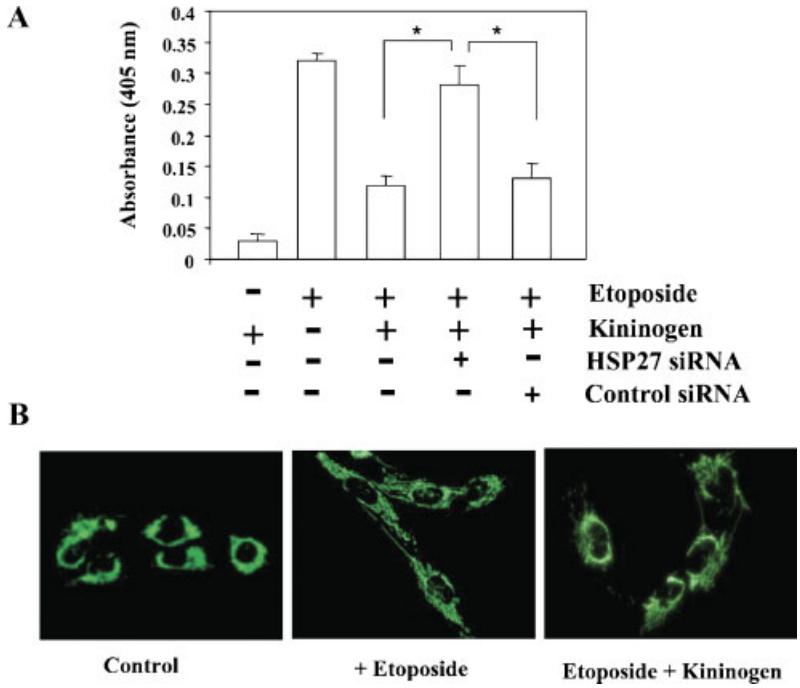

Fig. 6. Effect of KNG on etoposide-induced apoptosis in pagetic bone marrow stromal cells. **A**: Paget's bone marrow stromal cells were transiently transfected with HSP27 siRNA or control siRNA, and stimulated with or without KNG in the presence or absence of etoposide (25 μM) for 24 h. Total cell lysates obtained were assayed for caspase-3 activity was measured as described in methods. **B**: Immunofluorescence staining of cytochrome c distribution in KNG treated control cells, etoposide alone and KNG + etoposide combination treatment to pagetic marrow stromal cells.

in the presence or absence of etoposide (25 μM) and assayed for caspase-3 activation. As shown in Figure 6A, KNG treatment significantly inhibits etoposide-induced caspase-3 activity in pagetic stromal cells. Furthermore, siRNA suppression of HSP-27 expression significantly decreased KNG inhibition of etoposide-induced caspase-3 activity in these cells. Evident from the immunoflurenscence staining, etoposide treatment to pagetic marrow stromal cells demonstrated a diffused pattern of cytochrome c staining further confirms apoptosis in these cells. However, etoposide treatment in the presence of KNG (25 μM) demonstrates low levels of cytochrome c diffusion compared to etoposide alone treated cells (Fig. 6B). These results indicate functional role for HSP27 in KNG inhibition of apoptosis in pagetic marrow stromal/preosteoblastic cells.

DISCUSSION

Enhanced levels of IL-6, M-CSF, and endothelin-1 have been associated with PD, implicated in its pathogenesis and indicator of disease activity. Although PD is localized, bones not clinically involved with PD appear to show increased bone remodeling. For example, the increased levels of IL-6 in the peripheral blood of patients with PD may in part explain the increased bone remodeling seen in bones not clinically involved with PD. Therefore, it is necessary to define a pathologic role of systemic factors that are upregulated in patients with PD. In the present study, we have identified elevated levels of KNG expression in serum of patients with PD. Since KNG appears to be the major glycosylated peptide that is detected at the level of total protein analysis of patient's serum, we further determined KNG influence on pagetic bone marrow derived stromal/pre-osteoblast cells and associated signaling mechanism. The KNG is a multifunctional inflammatory cytokine which is composed of a 362-amino acid heavy chain, the 9-residue bradykinin sequence, and one 255-amino acid light chain [Takagaki et al., 1985]. KNG being a glycosylated secretory molecule, posttranslational regulatory mechanisms may be responsible for enhanced levels of serum KNG in patients with PD. The KNG localizes on the surface of endothelial cells, platelets, and

neutrophils. It has also been demonstrated that KNG purified from bovine milk stimulates proliferation of osteoblastic cells; however, the molecular signaling mechanism is unclear [Yamamura et al., 2000]. Although our results indicate KNG treatment results in high levels of HSP27 phosphorylation in pagetic stromal cells, we observe no significant change in RANKL expression in these cells. We have recently demonstrated that HSF-2 is a downstream target of fibroblast growth factor-2 (FGF-2) to induce RANKL expression in stromal/preosteoblast cells [Roccisana et al., 2004]. HSP are molecular chaperones activated upon cellular stress/stimuli [Snoeckx et al., 2001]. HSPs have been shown to prevent inflammatory damage through production of antiinflammatory cytokines [van Eden et al., 2005]. Several members of the *HSP* gene family have been reported to exhibit differential expression during stromal/preosteoblast differentiation. The differences in HSP expression are consistent with involvement in mediating a series of regulatory events functionally related to the physiologic control of cell growth and differentiation [Shakoori et al., 1992]. Our results using siRNA suppression of HSP27 did not significantly affect KNG stimulation of pagetic marrow stromal cell proliferation. However, inhibition of ERK phosphorylation completely abolished the KNG stimulated cell proliferation suggesting that KNG enhances proliferation of pagetic stromal cells through ERK signaling pathway. Therefore, KNG may play an important role in modulating marrow stromal cell proliferation/differentiation. Although KNG exerts similar effect on normal and pagetic marrow derived stromal cells with respect to HSP27 and ERK activation, our results indicate that KNG stimulates pagetic marrow derived stromal cell proliferation efficiently compared to normal bone marrow derived cells. It is possible that the pagetic marrow stromal cells are either indirectly or directly affected by the elevated systemic factors and chronic exposure to cytokines produced in the focal lesions. Alternatively, pagetic cells may be more sensitive to KNG stimulation due to an intrinsic genetic defect in patients with PD.

HSPs have been implicated with anti-apoptotic role in mammalian cells. Recent evidence further indicates that rapid phosphorylataion of HSP27 is required for cell adhesion and suppression of apoptosis in renal epithelial cells [de Graauw et al., 2005]. In the present study, KNG-induced HSP27 activation and suppression of etoposide-induced caspase-3 activity suggest antiapoptotic role for KNG in pagetic marrow stromal/preosteoblastic cells. In support of our results, recently Kaschina et al. (2004), demonstrated that KNG deficiency results in enhanced caspase-3 mediated cellular apoptosis. In summary, KNG modulate pagetic bone marrow stromal/preosteoblast cell proliferation through ERK signaling pathway and suppress etoposide-induced apoptosis through enhanced HSP27 phosphorylation. Therefore, enhanced levels of KNG in patients with PD further implicate a pathophysiologic role for KNG in PD.

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Expression of Measles Virus Nucleocapsid Protein in Osteoclasts Induces Paget's Disease-Like Bone Lesions in Mice

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ABSTRACT: We targeted the *MVNP* gene to the OCL lineage in transgenic mice. These mice developed abnormal OCLs and bone lesions similar to those found in Paget's patients. These results show that persistent expression of MVNP in OCLs can induce pagetic-like bone lesions in vivo.

Introduction: Paget's disease (PD) of bone is the second most common bone disease. Both genetic and viral factors have been implicated in its pathogenesis, but their exact roles in vivo are unclear. We previously reported that transfection of normal human osteoclast (OCL) precursors with the measles virus nucleocapsid (MVNP) or measles virus (MV) infection of bone marrow cells from transgenic mice expressing a MV receptor results in formation of pagetic-like OCLs.
Materials and Methods: Based on these in vitro studies, we determined if the *MVNP* gene from either an Edmonston-related strain of MV or a *MVNP* gene sequence derived from a patient with PD (P-MVNP), when targeted to cells in the OCL lineage of transgenic mice with the TRACP promoter (TRACP/MVNP mice), induced changes in bone similar to those found in PD.
Results: Bone marrow culture studies and histomorphometric analysis of bones from these mice showed that their OCLs displayed many of the features of pagetic OCLs and that they developed bone lesions that were similar to those in patients with PD. Furthermore, IL-6 seemed to be required for the development of the pagetic phenotype in OCLs from TRACP/MVNP mice.
Conclusions: These results show that persistent expression of the *MVNP* gene in cells of the OCL lineage can induce pagetic-like bone lesions in vivo.
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Key words: Paget's disease, osteoclast, TAF_{II}-17, IL-6, measles virus nucleocapsid

INTRODUCTION

Paget's disease (PD) of bone is the second most common bone disease, affecting 1–2 million patients in the United States. Although the etiology of PD is unknown, both genetic and nongenetic factors have been implicated. Studies of large families with PD have shown an autosomal dominant mode of inheritance, and recently, several loci have been linked to PD.[1–5] Mutations in the *sequestosome-1* (*SQSTM1*) gene occur in ~30% of patients with familial or 10% of patients with sporadic PD,[6] although the penetrance of PD in families with these mutations is variable. In addition, previous studies have suggested a viral etiology for PD. Electron microscopic studies first showed nuclear inclusions in pagetic osteoclasts (OCLs), which were similar to paramyxoviral nucleocapsids.[7] Immunohistochemical studies subsequently identified both respiratory syncytial virus and measles virus nucleocapsid proteins (MVNPs) in pagetic OCLs.[8] In situ hybridization studies also showed MVNP transcripts in cells from bone biopsy specimens from patients with PD,[9] and RT-PCR studies identified MVNP or canine distemper virus nucleocapsid transcripts in OCLs from patients with PD.[10,11] However, others have been unable to detect viral transcripts in pagetic OCLs.[12,13] Thus, the role of paramyxoviruses in the pathogenesis of PD is unclear.

We previously reported that transfection of normal human OCL precursors with the *MVNP* gene results in formation of OCLs that have many of the abnormal features of pagetic OCLs.[14] Both pagetic and MVNP-transfected normal OCL precursors form markedly increased numbers of OCLs in vitro, which contain many more nuclei per OCL and have an increased resorption capacity compared with normal OCLs. Furthermore, both pagetic and MVNP-transfected normal OCL precursors display marked hyperresponsivity to $1,25(OH)_2D_3$, forming OCLs at concentrations that are one to two logs lower than required for

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normal OCL formation.[15] In addition, both pagetic and MVNP-transfected OCL precursors express high levels of TAF_{II}-17, a member of the TF-IID transcription complex, which acts as a coactivator of vitamin D receptor–mediated gene transcription.[16] OCL from PD patients and OCL precursors transduced with the *MVNP* gene also secrete large amounts of IL-6.[14,17] Finally, when bone marrow cells from transgenic mice in which the CD46 MV receptor[18] is targeted to cells in the OCL lineage are infected in vitro with MV, they form OCLs that have the abnormal characteristics of pagetic OCLs.[19] However, it is unknown if MV can induce pagetic-like bone lesions in vivo that are similar to the abnormal bone present in PD.

In this study, we determined if persistent expression of the nucleocapsid gene from either an Edmonston variant of MV (E-MVNP) or the nucleocapsid sequence derived from a patient with PD (P-MVNP)[20] could induce changes in bone similar to those found in PD. The *E-MVNP* or *P-MVNP* gene was targeted to cells in the OCL lineage in transgenic mice using the TRACP promoter. These mice were analyzed at 4–16 months of age to determine if they developed bone abnormalities similar to those seen in PD.

MATERIALS AND METHODS

Development of TRACP/E-MVNP and TRACP/P-MVNP transgenic mice

These studies were approved by the IACUCs at both the University of Pittsburgh School of Medicine and Virginia Commonwealth University. The E-MVNP cDNA, originally derived from a measles patient, was generously provided by Dr Chris Richardson of the University of Toronto. Sequence analysis of this cDNA showed that it was from a virus belonging to the Edmonston group of MV strains, which is the most widespread group of MV strains and the origin of the majority of MV vaccines.[21] This cDNA encodes a protein that differs from the Edmonston strain wildtype MVNP (GI: 1041617)[22] at five amino acid residues: 26 (G to E), 453 (E to G), 467 (L to P), 473 (L to P), and 525 (D to G). Sequence analysis of the *P-MVNP* gene showed that it also encodes a closely related Edmonston strain MVNP that differs from the wildtype Edmonston MVNP at seven residues: 26 (G to E), 435 (K to R), 453 (E to G), 467 (L to P), 473 (L to P), 494 (A to T), and 525 (D to E). The majority of the amino acid differences between either the E-MVNP or P-MVNP and the wildtype MVNP fall within the hypervariable carboxy terminus of this protein. When we initially reported the detection of MVNP transcripts from the bone marrow of several Paget's patients,[20] the sequence from patient 1 (P1) diverged from the consensus MVNP sequence beginning at amino acid 497. It was subsequently determined that this apparent divergence was caused by a DNA sequencing error that produced a frameshift in P1 relative to the consensus MVNP sequence and that the P1 sequence in fact matches wildtype MVNP with the exceptions noted above. To generate the *TRACP/E-MVNP* and *TRACP/P-MVNP* transgenes, the E-MVNP and P-MVNP cDNA was inserted into the unique *Eco*RI site of the pBSmTRACP5′ plasmid.[23,24] This resulted in the addition of a 25 amino acid C-terminal tag to the P-MVNP but not the E-MVNP construct. The transgenes were excised with *Xho*I, and transgenic mice were generated by standard methods[25] in a CB6F1 (C57Bl/6 × Balb/c) genetic background. Transgenic founders were identified by Southern blot analysis of tail DNA, and transgenic mice of subsequent generations were identified by PCR analysis. Two TRACP/E-MVNP and four TRACP/P-MVNP founder mice were generated and bred to establish multiple independent lines of mice. OCL formation assays were performed on marrow cultures from all transgenic lines, and the TRACP/E-MVNP and TRACP/P-MVNP line that had the highest level of MVNP expression and OCL formation were selected for further characterization and longitudinal studies. This was necessitated by the large numbers of mice that had to be maintained and the extensive histomorphometric analysis that was required. All data presented here were derived from one line for each transgene that expressed the highest levels of the transgene, although increased levels of OCL formation and hypersensitivity to $1,25(OH)_2D_3$ were found in marrow cultures from the additional transgenic lines.

Immunohistochemical detection and Western blot analysis of MVNP expression in marrow cells from TRACP/MVNP and WT mice and PD patients

OCLs from nonadherent mouse bone marrow cells (2×10^5 cells/well) from TRACP/MVNP or WT mice cultured for 7 days with 10^{-8} M $1,25(OH)_2D_3$ were tested for cross-reactivity with a monoclonal antibody against the MVNP protein (Gene Tex, San Antonio, TX, USA) or mouse IgG (60 ng/ml) using a Vectastatin-ABC-AP kit (Vector Laboratories, Burlingame, CA, USA) as previously described.[19] For Western blot analysis, nonadherent mouse bone marrow cells (1.2×10^6 cells) from TRACP/MVNP or WT mice were cultured with macrophage-colony stimulating factor (M-CSF; 10 ng/ml)/RANKL (25 ng/ml) in α-MEM–10% FBS for 48 h. Cell lysates were prepared and processed for Western blot analysis as previously described.[14] The MVNP monoclonal antibody or β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at 1:1000 dilution in Tris-buffered saline containing 1% BSA. Nonadherent marrow mononuclear cells (5×10^5 cells) from patients with PD or normals were cultured for 3 weeks with 10^{-8} M $1,25(OH)_2D_3$, to induce OCL formation, and the cells were processed for Western blot analysis as described above.

PCR analysis for TAF_{II}-17 expression

MVNP and WT mice OCL precursors were cultured for 2 days with $1,25(OH)_2D_3$ (10^{-8} M) and subjected to RT-PCR for TAF_{II}-17 mRNA as previously described.[16] The mouse TAF_{II}-17 sense primer was 5′-CAGATTATGAACCAGTTTGGCCCTTCA-3′ and was derived from the *TAF_{II}-17* gene sequence (GenBank accession no. AL590442). The TAF_{II}-17 antisense primer was 5′-CCTGTGTTATTTCTTGGTTGTTTTCCG-3′. The actin sense and antisense primers were 5′-GGCCGTACCACTGGCATCGTGATG-3′ and 5′-CLTGGCCGTCAGGCAGCTCGTAGC-3′ and were derived from the actin gene sequence (GenBank accession no. NM000492).

In vitro analysis of OCL formation by bone marrow cells from TRACP/MVNP or WT mice

Nonadherent marrow cells from long bones of TRACP/E-MVNP, TRACP/P-MVNP, or WT mice at 4–16 months of age were cultured for OCL formation in the presence of varying concentrations of $1,25(OH)_2D_3$ (Roche, Indianapolis, IN, USA) or with 10 ng/ml of murine M-CSF and 25 ng/ml RANKL (R&D Systems, Minneapolis, MN, USA) for 7 days as previously described.[26] The cells were stained for TRACP activity using a commercial kit (Sigma, St Louis, MO, USA), and the number of $TRACP^+$ multinucleated cells that contained at least three nuclei, as well as the number of nuclei per multinucleated cell, were scored microscopically. IL-6 levels in conditioned media from these marrow cultures were determined using a commercial ELISA kit (R&D Systems). In selected experiments, marrow cultures were treated with vehicle or $1,25(OH)_2D_3$ in the presence of a neutralizing antibody to murine IL-6 (50 ng/ml) or isotype specific mouse IgG (R&D Systems) to assess the effects of IL-6 on OCL formation.

Histologic analysis of TRACP/E-MVNP and TRACP/P-MVNP vertebral bones

The first to fourth lumbar vertebrae from TRACP/E-MVNP, TRACP/P-MVNP, and WT mice were fixed in 10% buffered formalin and completely decalcified in 10% EDTA at 4°C and embedded in paraffin. Five-micrometer longitudinal sections were cut and mounted on glass slides. Deparaffinized sections were stained for TRACP as described by Liu et al.[27] OCLs containing active TRACP were stained red. Another set of sections was stained with 0.1% toluidine blue.

Histomorphometry was performed on the region of cancellous bone between the cranial and caudal growth plates of the third lumbar vertebral body under bright field and polarized light at a magnification of ×200, using the OsteoMeasure 4.00C morphometric program (OsteoMeasure; OsteoMetrics, Atlanta, GA, USA). Osteoclast perimeter (Oc.Pm) was defined as the length of bone surface covered with $TRACP^+$ mono- and multinuclear cells. Osteoblast perimeter (Ob.Pm), cancellous bone volume (BV/TV), trabecular width (Tb.Wi), trabecular number (Tb.N), and trabecular separation (Tb.Sp) were also quantified and calculated.

To examine bone formation parameters, animals were given calcein (10 mg) subcutaneously on days 7 and 1 before death. Bones from these animals were embedded, undecalcified, in methyl methacrylate and sections were examined under fluorescent light for quantification of mineralized perimeter, mineral apposition rate, and bone formation rate. All variables were expressed and calculated according to the recommendations of the ASBMR Nomenclature Committee.[28,29]

Statistical analysis

In vitro culture results were analyzed by a two-way ANOVA. Histomorphometric variables were analyzed by two-factor ANOVA using NCSS 2004 software (NCSS Statistical Software, Kaysville, UT, USA). Genotype and age were assigned as factors and the responses of the measured variables were tested. A p value of <0.05 was considered statistically significant.

RESULTS

Analysis of OCLs from TRACP/E-MVNP and TRACP/P-MVNP mice for expression of MVNP

Immunohistochemical analysis showed that OCL formed in marrow cultures from TRACP/E-MVNP and TRACP/P-MVNP mice expressed MVNP (Fig. 1A). Similar levels of staining were detected in both the TRACP/E-MVNP and TRACP/P-MVNP OCLs, and no staining was seen in OCLs from nontransgenic control (WT) or normal human marrow cultures. These results were confirmed by Western blot analysis of MVNP expression, which showed expression of MVNP in TRACP/MVNP and PD patient samples but not in WT mice or normal marrow (Figs. 1B and 1C). Importantly, the levels of MVNP expression in both lines of transgenic mice was roughly comparable with that seen in OCL formed from marrow cultures of patients with PD (Fig. 1C).

Characterization of OCLs formed in marrow cultures of TRACP/E-MVNP and TRACP/P-MVNP mice

Significantly more OCLs were formed in marrow cultures from TRACP/E-MVNP and TRACP/P-MVNP mice than from WT mice in response to $1,25(OH)_2D_3$ (Fig. 2A). Furthermore, both TRACP/E-MVNP and TRACP/P-MVNP marrow cultures formed OCLs at concentrations of $1,25(OH)_2D_3$ that were significantly lower than those required for WT marrow cultures, with OCL formation occurring in MVNP cultures at 10^{-11} to 10^{-12} M $1,25(OH)_2D_3$. In addition, the OCL precursors from TRACP/P-MVNP and E-MVNP, but not WT mice, expressed high levels of TAF_{II}-17 mRNA (Fig. 1D). The number of nuclei per OCL was also significantly increased in marrow cultures from TRACP/E-MVNP and TRACP/P-MVNP mice compared with WT mice (Fig. 2B), and the OCLs that formed were larger than those formed in WT marrow cultures (Fig. 2C). In contrast, there was no significant difference in the sensitivity of OCL precursors from WT, TRACP/E-MVNP, and TRACP/P-MVNP mice to RANKL or expression of RANK mRNA in OCL precursors from these mice (data not shown).

IL-6 production and effects of anti-IL-6 in bone marrow cultures from TRACP/P-MVNP or WT mice

Low levels of IL-6 were detected in bone marrow–conditioned media from marrow cultures of TRACP/P-MVNP and WT mice treated with vehicle. In contrast, high levels of IL-6 were present in conditioned media of cultures of TRACP/P-MVNP, but not WT mice, treated with $1,25(OH)_2D_3$ to induce OCL formation (Fig. 3A). In contrast, IL-11 levels were similar in conditioned media from WT and TRACP/P-MVNP or TRACP/E-MVNP marrow cultures (data not shown).

We determined if IL-6 also played a role in the formation of pagetic-like OCLs in TRACP/P-MVNP marrow cultures. Addition of an anti-IL-6 antibody (50 ng/ml) to marrow cultures of TRACP/P-MVNP mice treated with $1,25(OH)_2D_3$ significantly decreased OCL numbers and the number of nuclei per OCL (Fig. 3B; Table 1). In contrast,

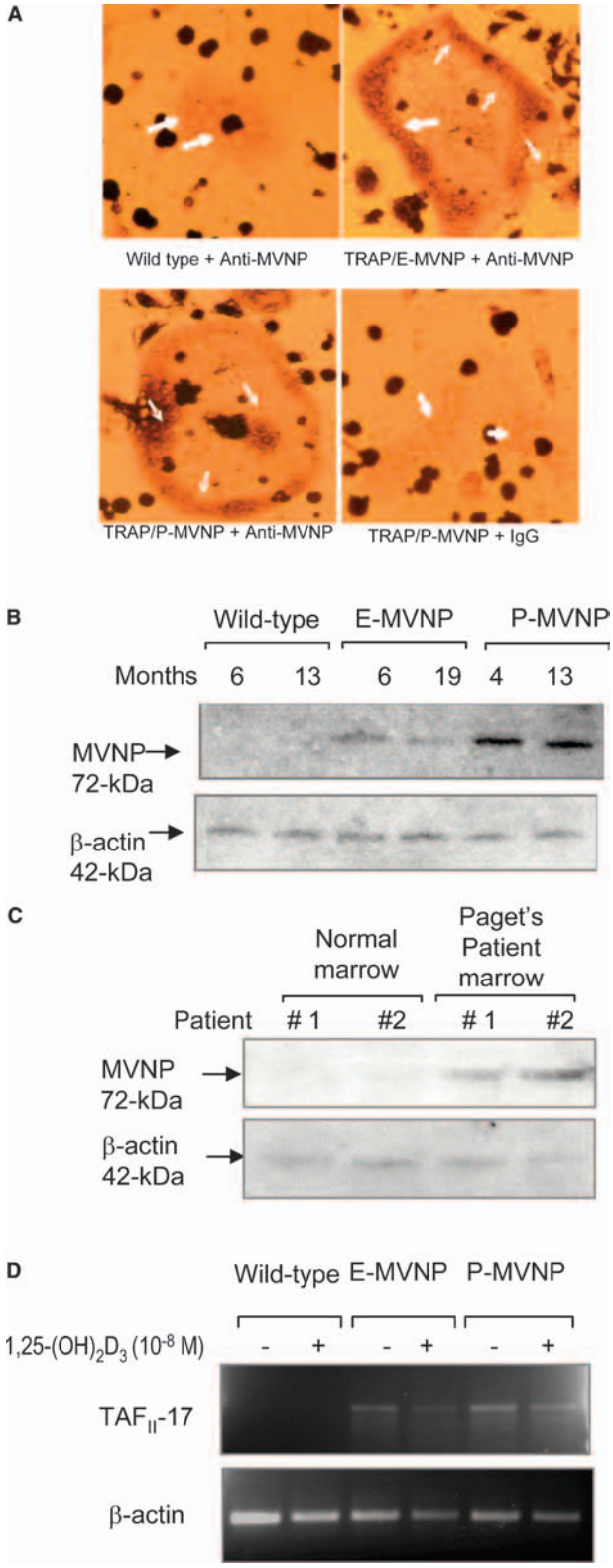

FIG. 1. (A) Expression of MVNP in OCLs formed from TRACP/MVNP mice. Nonadherent mouse bone marrow cells from WT, TRACP/E-MVNP, or TRACP/P-MVNP mice were cultured for 7 days with 10^{-8} M $1,25(OH)_2D_3$ and tested for cross-reactivity with a monoclonal antibody against the MVNP protein or control IgG using a Vectastatin-ABC-AP kit. Mouse IgG (60ng/ml) was used as a negative control. Arrows denote nuclei. Cross-reactivity with the anti-MVNP results in nuclei that are stained brown. The dark particles throughout are precipitated alkaline phosphatase stain. Magnification, ×100. (B) Western blot analysis of MVNP expression by nonadherent mouse bone marrow cells from TRACP/MVNP and WT mice and (C) MVNP in OCLs from marrow cultures from normal individuals and patients with PD. Lysates were prepared and analyzed. The gels were reprobed for β-actin expression to control for loading. (D) TAF_{II}-17 mRNA expression by MVNP and WT mice OCL precursors. MVNP and WT mice OCL precursors were cultured for 2 days with $1,25(OH)_2D_3$ (10^{-8} M) and subjected to RT-PCR analysis using the primers listed for TAF_{II}-17 or actin mRNA. The conditions for amplification were as follow: 94°C for 5 minutes, 35 (TAF_{II}-17) or 28 cycles (β actin) at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, followed by extension at 72°C for 7 minutes. PCR products were separated by 2% agarose gel electrophoresis.

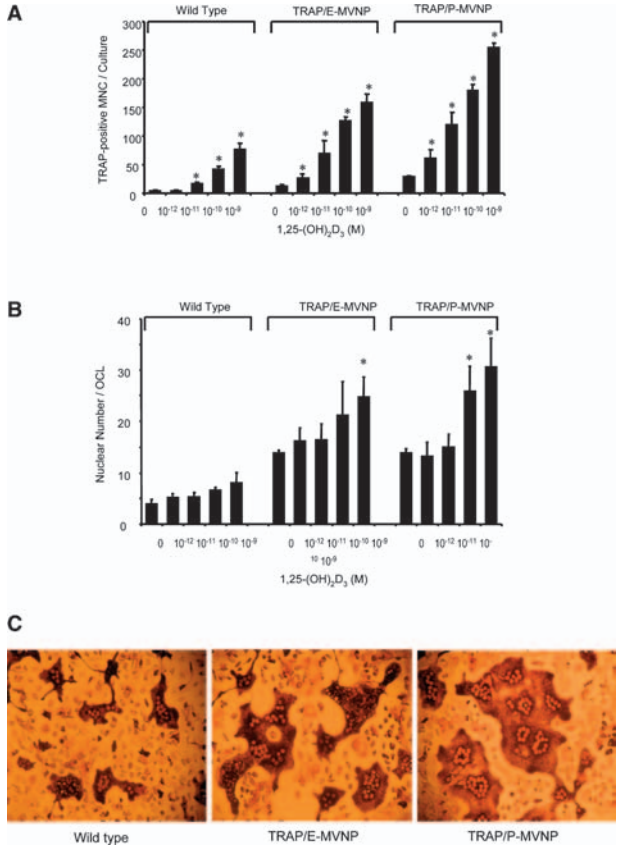

FIG. 2. OCLs formed in marrow cultures from TRACP/MVNP mice. (A) OCL formation in TRACP/MVNP mice. OCL precursors (10^5 cells/well) from WT, TRACP/E-MVNP, or TRACP/P-MVNP mice were cultured in the presence of varying concentrations of $1,25(OH)_2D_3$. After 7 days of culture, the cells were fixed with 1% formaldehyde and stained for TRACP using a leukocyte acid phosphatase kit (Sigma). The $TRACP^+$ multinucleated cells were scored using an inverted microscope. The results are expressed as mean ± SE for quadruplicate cultures from a typical experiment. *Significantly different from marrow cultures treated with vehicle alone ($p < 0.01$). Similar results were obtained in five independent experiments. (B) Nuclear number per $TRACP^+$ OCLs. The number of nuclei per OCL was determined in 20 random $TRACP^+$ OCLs for each treatment group, and the results are expressed as mean ± SE. *Significantly different from cultures treated with vehicle alone ($p < 0.01$). (C) Morphology of multinucleated cells formed from TRACP-MVNP mice by OCL precursors treated with 10^{-8} M $1,25(OH)_2D_3$ for 9 days and stained for TRACP.

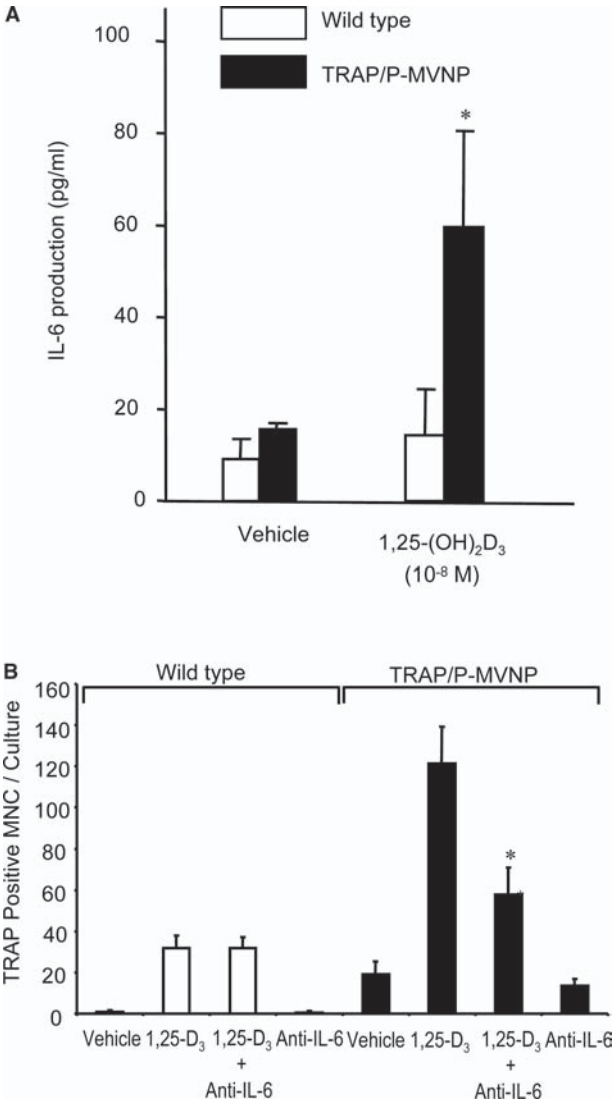

FIG. 3. (A) IL-6 production and (B) effects of anti-IL-6 on bone marrow cultures from TRACP/MVNP or WT mice. (A) Mouse bone marrow cells were treated with vehicle (media alone) or 10^{-8} M $1,25(OH)_2D_3$ for 7 days. Conditioned media were harvested at 7 days, and the concentration of IL-6 was determined using ELISA kits for mouse IL-6 (R&D Systems). Results are reported as IL-6 concentration (pg/ml) and are the mean ± SD of triplicate samples. A similar pattern of results was seen in two independent experiments. *Significantly different from WT marrow treated with the same concentration of $1,25(OH)_2D_3$ ($p < 0.001$). (B) Bone marrow cells (10^5 cells/well) from TRACP/P-MVNP or WT mice were cultured with 10^{-8} M $1,25(OH)_2D_3$ in the presence or absence of 50 ng/ml anti-mouse IL-6 or isotype specific mouse IgG (R&D Systems). This concentration of anti-IL-6 can neutralize 5 ng/ml of IL-6. After 7 days of culture, the cells were fixed with 1% formaldehyde and stained for TRACP using a leukocyte acid phosphatase kit (Sigma). The $TRACP^+$ multinucleated cells were scored using an inverted microscope. The results are expressed as mean ± SE for quadruplicate cultures from a typical experiment. *Significantly different from TRACP/P-MVNP cultures treated with $1,25(OH)_2D_3$ alone ($p < 0.001$). Similar results were obtained in three independent experiments.

anti-IL-6 had no effect on OCL formation or nuclear number per OCL in WT bone marrow cultures (Fig. 3B; Table 1).

TABLE 1. EFFECTS OF ANTI-IL-6 ON NUCLEAR NUMBER/OCLS IN TRACP/MVNP MICE

Cell type	*Vehicle*	*Anti-IL-6 (50 ng/ml)*	*$1,25(OH)_2D_3$ (10^{-8} M)*	*$1,25(OH)_2D_3$ (10^{-8} M) + anti-IL-6 (50 ng/ml)*
WT mice	3 ± 1	4 ± 1	4 ± 1	4 ± 1
TRACP/P-MVNP	6 ± 1	5 ± 1	16 ± 5	6 ± 1*

Bone marrow cells from TRACP/P-MVNP or WT mice were treated with or without $1,25(OH)_2D_3$ and anti-IL-6. At the end of the culture period, the cells were stained for TRACP activity, and the number of nuclei per OCL was assessed in 15 random $TRACP^+$ OCLs for each treatment group, in quadruplicate cultures, from two independent experiments. The results are expressed as mean ± SD.

* Significantly different from cultures treated with $1,25(OH)_2D_3$ ($p < 0.01$).

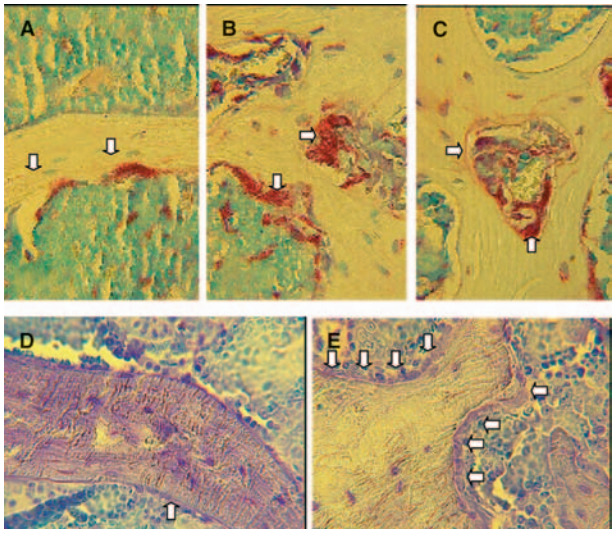

FIG. 4. OCL morphology in TRACP/MVNP and WT controls. OCL morphology in (A) WT controls and (B and C) TRACP/MVNP. Note larger OCLs in deeper resorption cavities and tunneling resorption in (B and C) MVNP (arrows) compared with fewer, smaller OCL in shallow resorption cavities in (A) WT controls (arrows). Original magnification, ×400. The number of mice analyzed is shown in Table 2. Osteoblast morphology in TRACP/MVNP and WT controls. Osteoblast morphology in (D) WT controls and (E) TRACP/MVNP. Note increased numbers of plump, cuboidal osteoblasts (arrows) in (D) TRACP/MVNP bones compared with fewer, flattened osteoblasts (arrow) in (E) WT bone. Original magnification, ×400.

Histology and histomorphometry of bones from TRACP/E-MVNP and TRACP/P-MVNP mice

There were no significant differences in the measured histomorphometric variables between bones from TRACP/E-MVNP and P-MVNP mice, and their histological features were qualitatively very similar. The data from these two groups were therefore pooled as one MVNP group and compared with WT animals.

MVNP OCLs were larger in size and had more nuclei per cell and the resorption cavities were deeper in MVNP bone

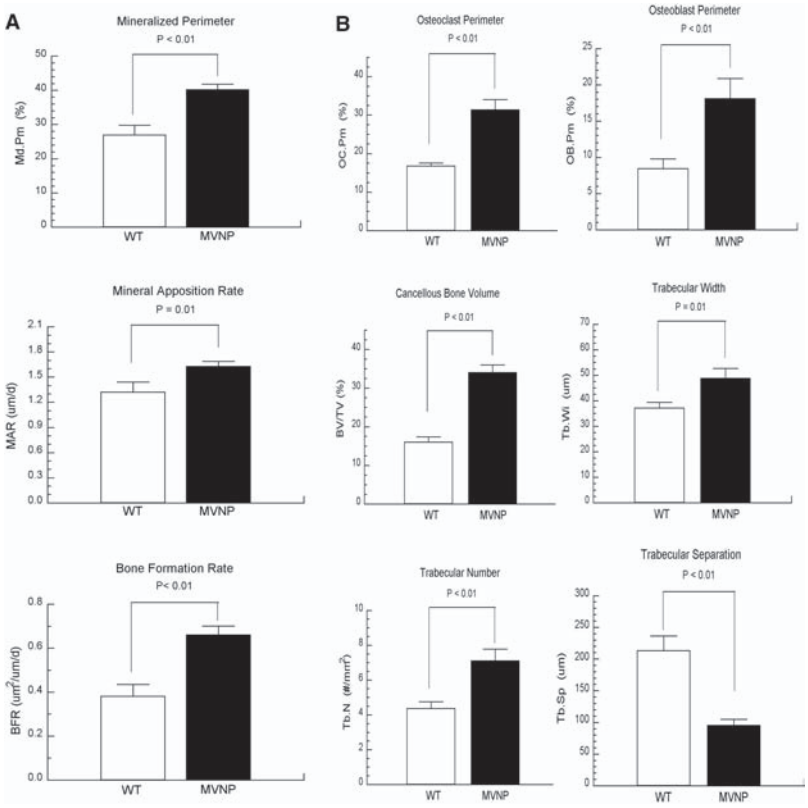

FIG. 5. (A) Mineralized perimeter, mineral apposition rate, and bone formation in 12 MVNP (age, 4–20 months; mean age, 13 months) and 10 WT mice (age, 4–19 months; mean age, 9 months). Data are expressed as mean ± SE. Note increased mineralized perimeter, mineral apposition rate, and bone formation rate in the MVNP mice. (B) OCL perimeter, osteoblast perimeter, cancellous bone volume, trabecular width, trabecular number, and trabecular separation in 4 12-month-old MVNP mice compared with 12 age-matched WT mice. Data are expressed as mean ± SE. Note increased OCL perimeter, osteoblast perimeter, cancellous bone volume, trabecular width, and trabecular number, but decreased trabecular separation in the MVNP mice. Data from all 14 12-month-old MVNP mice, including the 4 shown here, are shown in Table 3.

than in WT bone. Furthermore, tunneling resorption was present in MVNP bone but was rarely seen in WT bone (Figs. 4A–4C). Plump, cuboidal osteoblasts were more common in the MVNP than in the WT bone (Figs. 4D and 4E). Dynamic histomorphometric variables from nonlesioned bone in the calcein-labeled animals are shown in (Fig. 5A). Mineralized perimeter, mineral apposition rate, and bone formation rate were all significantly higher in the MVNP mice than in WT controls.

Markedly abnormal bone structure was seen in at least two of the four vertebrae examined in a subset of 4 of the 14 MVNP mice (29%) at 12 months of age. Two of these animals were TRACP/E-MVNP mice and two were TRACP/P-MVNP mice. These lesions were histologically similar to those seen in PD and were characterized by focally thickened and irregular trabeculae composed mainly of woven bone (Fig. 6). Cancellous bone volume, trabecular number, trabecular width, OCL perimeter, and osteoblast perimeter were all significantly increased in these four animals compared with age-matched WT controls, whereas trabecular separation was significantly reduced (Fig. 5B). None of these histological features was seen in WT controls.

To determine whether the dramatic changes seen in the vertebrae from these four animals were localized to individual vertebrae, we measured the histomorphometric variables in adjacent vertebrae that, qualitatively, did not appear to be as severely affected. These data are shown in Table 2. Whereas bone microarchitecture and turnover variables were not as abnormal in the adjacent vertebrae, they were still significantly different from those in WT animals. Data from the animals studied at 4, 8, and 12 months of age, including the lesioned bone from the four 12-month-old mice in Fig. 6, are given in Table 3. OCL perimeter was increased by 20–58% in MVNP mice compared with those from WT, and osteoblast perimeter was increased by 26–61%. The magnitude of the differences between MVNP and WT in OCL and osteoblast perimeters increased with age.

DISCUSSION

In this study, we determined the capacity of two different *MVNP* genes, one from an Edmonston group of MV originally isolated from a measles patient and one derived from a patient with PD, to induce a Paget's-like phenotype in transgenic mice. Expression of the two nucleocapsid genes was directed to cells in the OCL lineage using the TRACP promoter, which is highly expressed in OCLs and OCL precursors and has been used previously to target expression of multiple genes to cells in the OCL lineage.[30] TRACP is also expressed in chondrocytes and occasional osteoblasts in bone[31] but at very low levels compared with OCLs.

OCL precursors from TRACP/E-MVNP and TRACP/P-MVNP mice were found to be similar to each other and express almost all the features of pagetic OCL precursors. These include increased levels of OCL formation and a marked hyper-responsivity to $1,25(OH)_2D_3$. In addition, the OCLs that form are larger and contain many more

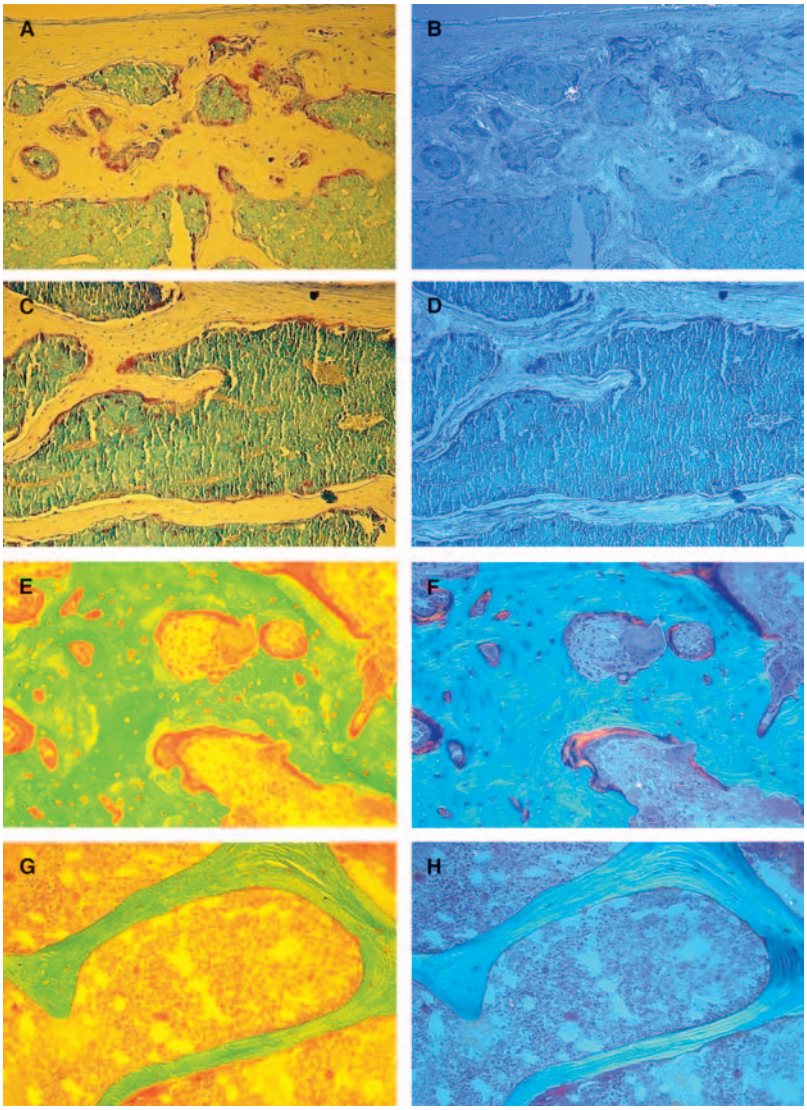

FIG. 6. Histological features of a 12-month-old TRACP/E-MVNP mouse compared with WT control. Histological features in (A and B) a TRACP/E-MVNP mouse compared with (C and D) WT controls at 12 months of age. Note thickened, irregular trabeculae, increased OCL number, (A) tunneling resorption, and (B) increased amounts of woven bone in the TRACP/MVNP mouse compared with the (C and D) WT control. Provided for comparison are sections from (E and F) a 58-year-old woman with PD and (G and H) a 58-year-old normal subject. (A and C) TRACP stain, counterstained with methyl green-thionin. (C and D) Same sections viewed under polarized light to reveal woven bone. (E and G) Goldner's trichrome stain. (G and H) Same sections viewed under polarized light. Original magnification, ×100.

nuclei per OCL. The only phenotypic difference that distinguished OCL precursors from TRACP/E-MVNP or TRACP/P-MVNP mice from OCL precursors from PD patients is that TRACP/MVNP OCL precursors are not hyper-responsive to RANKL.[32,33] These data suggest that additional factors, possibly genetic factors linked to PD, may be responsible for the hyper-responsivity of pagetic OCLs to RANKL.

Importantly, bones from the TRACP/E-MVNP or TRACP/P-MVNP mice displayed many of the histologic and histomorphometric features of bone lesions from patients with PD. These include an increase in mineralized perimeter, mineral apposition rate, bone formation rate, an increase in OCL and osteoblast perimeters, increases in the number and size of OCLs with more nuclei/OCL, deeper resorption cavities and tunneling resorption, and abundant large cuboidal osteoblasts. Furthermore, the bone that was formed was abnormal and was woven in character, similar to that seen in pagetic lesions. These changes were particu-

TABLE 2. CANCELLOUS BONE IN 12-MONTH MVNP AND WT MICE

Variables	*WT (n = 12)*	*MVNP lesion (n = 4)*	*MVNP adjacent (n = 4)*
BV/TV (%)	16.0 ± 1.4	34.1 ± 1.9*	24.9 ± 1.1*
Tb.Wi (μm)	37.1 ± 2.2	48.7 ± 4.0*	45.8 ± 2.7
Tb.N (#/mm^2)	4.4 ± 0.4	7.1 ± 0.7*	5.5 ± 0.2
Tb.Sp (μm)	213.2 ± 22.8	95.4 ± 9.3*	137.8 ± 5.9
Oc.Pm (%)	16.8 ± 0.8	31.4 ± 2.7*	28.3 ± 1.9*
Ob.Pm (%)	8.5 ± 1.4	19.9 ± 1.8*	17.8 ± 2.6*

Data are expressed as mean ± SE. The data were analyzed using ANOVA with Duncan's multiple-comparison.

* $p < 0.01$ vs. WT. MVNP data were pooled from two TRACP/E-MVNP and two TRACP/P-MVNP mice.

MVNP lesion, data from vertebrae with Paget's-like lesion; MVNP adjacent, data from vertebrae adjacent to those with Paget's-like lesion; BV/TV, cancellous bone volume; Tb.W, trabecular width; Tb.N, trabecular number; Tb.Sp, trabecular separation; Oc.Pm, osteoclast perimeter; Ob, osteoblast perimeter.

TABLE 3. CANCELLOUS BONE STRUCTURE IN MVNP AND WT MICE

Variables	*WT at 4 months (n = 18)*	*MVNP at 4 months (n = 17)*	*WT at 8 months (n = 12)*	*MVNP at 8 months (n = 10)*	*WT at 12 months (n = 12)*	*MVNP at 12 months (n = 14)*
BV/TV (%)	18.3 ± 0.8	16.9 ± 1.2	17.1 ± 1.9	17.8 ± 0.8	16.0 ± 1.4	20.4 ± 2.6
Tb.Wi (μm)	34.0 ± 1.0	31.9 ± 1.1	35.0 ± 2.28	36.7 ± 1.1	37.1 ± 2.2	39.9 ± 2.3
Tb.N (#/mm^2)	5.4 ± 0.2	5.3 ± 0.3	4.8 ± 0.4	4.9 ± 0.2	4.4 ± 0.4	5.0 ± 0.5
Tb.Sp (μm)	155.7 ± 6.7	163.9 ± 9.7	184.7 ± 24.1	171.8 ± 7.0	213.2 ± 22.8	182.8 ± 21.9
Oc.Pm (%)	17.5 ± 1.1	21.0 ± 0.8*	17.0 ± 1.3	21.3 ± 1.3*	16.8 ± 0.8	26.5 ± 1.9*
Ob.Pm (%)	11.6 ± 1.1	14.6 ± 1.3*	6.9 ± 1.0	10.1 ± 1.2*	8.5 ± 1.4	13.6 ± 1.5*

Data are expressed as mean ± SE.
See Table 2 for abbreviations.
* Significant differences between MVNP and MVNP ($p < 0.01$, by two-factor ANOVA).

larly evident in 30% of the animals at 12 months of age. In addition to the marked increase in turnover, the lesions in these animals displayed a dramatic increase in bone volume and trabecular thickness. The abnormally thickened and coarse trabeculae were remarkably similar to those seen in PD, and the fact that these dramatic lesions were only observed in the oldest animals is also consistent with the slow development of pagetic lesions. Furthermore, not all of the bones in these animals were as severely affected, consistent with a variable rate of expression of the phenotype in different bones, although adjacent bones showed increased OCL and osteoblast activity (Table 2). These data suggest that the rate of development of the pagetic-like lesions differed in the individual vertebral from these mice. In contrast to these findings, high turnover states are generally associated with reduced, rather than increased, bone volume in both humans and experimental animals. For example, transgenic mouse models of both primary and secondary hyperparathyroidism display increased resorption and formation, but bone volume is reduced.[34,35] Also, unlike the histological changes accompanying chronic PTH excess,[36] the MVNP mice did not show any peritrabecular or marrow fibrosis. Increased bone volume does accompany high bone turnover states, but only when the stimulus is anabolic (e.g., intermittent, exogenous administration of PTH or PGE_2).[37,38] Woven bone formation is also seen under such circumstances but requires very high doses.[37,38] Furthermore, such anabolic effects occur throughout the skeleton, rather than being restricted to individual bones, as seen in the TRACP/MVNP mice. Furthermore, other transgenic mouse models in which the TRACP promoter has been used to target genes to the OCL lineage do not develop pagetic-like bone lesions similar to those found in TRACP/MVNP mice.[30] When taken together, these observations strongly suggest that the bone lesions observed in the MVNP mice are the result of osteoclastic expression of the nucleocapsid genes rather than being caused by a generalized high turnover state.

We previously hypothesized that the sequence variants of the MVNP transcripts isolated from Paget's patients might contribute to the unique pathogenic role of MVNP in PD.[20] However, in this study, we introduced two MVNP variants into transgenic mice, one originally derived from a MV patient and one derived from a Paget's patient, and the resulting phenotype is indistinguishable. The majority of the amino acid substitutions between either E-MVNP or P-MVNP and the Edmonston WT MVNP, as well as the differences between E-MVNP and P-MVNP, fall within the C-terminal region of the protein, which is known to be hypervariable.[21] Thus, it now seems unlikely that the unique amino acid substitutions seen in P-MVNP specifically contribute to its pathogenic role in PD.

The *MVNP* gene can have effects on other human cells in addition to OCLs. During initial infection by MV, transient profound immune suppression occurs, followed by development of long-term immunity to MV.[39] The mechanism for this immunosuppression involves binding of the nucleocapsid protein to the Fc-γ receptors on dendritic cells, resulting in suppression of IL-12 expression and increased IL-6 expression.[40] These data suggest that MVNP can have profound effects on cellular function in cells of the monocyte macrophage lineage, and it is the same precursor cell in this lineage that gives rise to both OCL and dendritic cells. Thus, it is reasonable that the OCL precursors in the monocyte macrophage lineage could be affected by MV.

The mechanism(s) underlying the capacity of the *MVNP* gene to induce pagetic-like OCLs in vitro and in vivo are still being defined. Previous reports have shown that chronic infection of human glial cells with MV markedly increases IL-6 production with little or no increase in IL-1β or TNF-α expression.[41] Induction of IL-6 expression in OCL seems to play a role in the abnormal OCLs formed in TRACP/MVNP mice. lL-6 levels were increased in conditioned media from marrow cultures of TRACP/P-MVNP mice induced to form OCLs, and an anti-IL-6 antibody decreased both OCL formation and nuclei/OCL in TRACP/P-MVNP marrow cultures. In contrast, IL-6 levels were not increased in WT marrow cultures treated with $1,25(OH)_2D_3$, and anti-IL-6 had no effect on OCL formation in WT cultures. We previously showed that OCLs formed in cultures of marrow from PD patients also produce high levels of IL-6.[17] Taken together, these data support an important role for IL-6 in the abnormal OCL formation in PD and suggest that MVNP may be responsible for the increased IL-6 expression in pagetic OCLs. These results further suggest that other factors yet to be defined are involved in the abnormal OCL formation in TRACP/MVNP mice because anti-IL-6 did not reduce OCL formation to control levels.

However, this factor does not seem to be IL-11, because IL-11 levels were not increased by MVNP expression in OCL precursors.

Although the experiments reported here do not prove that MV can cause PD, they clearly show that expression of MVNP in cells of the OCL lineage can result in bone lesions and abnormalities in OCL precursors that are very similar to those found in patients with PD. In addition, just as in patients with a genetic predisposition to PD (e.g., an inherited *SQSTM1* mutation), the development of PD is variable, not all of the mice in our cohorts developed PD-like lesions. Furthermore, like patients with PD, the lesions that did arise were focal despite the forced expression of MVNP in the majority of OCLs in the transgenic mice.

Thus, persistent expression of MVNP in cells of the OCL lineage can induce pagetic-like lesions in vivo. These results suggest that persistent expression of MVNP in OCLs is an important component in the complex etiology of PD.

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